

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12N 15/62, C07K 15/12, C12N 1/21	A1	(11) International Publication Number: <b>WO 94/26910</b> (43) International Publication Date: 24 November 1994 (24.11.94)
(21) International Application Number: PCT/US94/05348 (22) International Filing Date: 12 May 1994 (12.05.94) (30) Priority Data: 08/064,691                      12 May 1993 (12.05.93)                      US (71) Applicant (for all designated States except US): XOMA CORPORATION [US/US]; 2910 7th Street, Berkeley, CA 94710 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): BETTER, Marc, D. [US/US]; 2462 Zorada Drive, Los Angeles, CA 90046 (US). CARROLL, Stephen, F. [US/US]; 1308 Milton Avenue, Walnut Creek, CA 94596 (US). STUDNICKA, Gary, M. [US/US]; 1504 Berkeley Street, Santa Monica, CA 90404 (US). (74) Agent: NOLAND, Greta, E.; Marshall, O'Toole, Gerstein, Murray and Borun, 6300 Sears Tower, 233 South Wacker Drive, Chicago, IL 60606 (US).	(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	

(54) Title: IMMUNOTOXINS COMPRISING GELONIN AND AN ANTIBODY

## (57) Abstract

The present invention provides purified and isolated polynucleotides encoding Type I ribosome-inactivating proteins (RIPs) and analogs of the RIPs having a cysteine available for disulfide bonding to targeting molecules. Vectors comprising the polynucleotides and host cells transformed with the vectors are also provided. The RIPs and RIP analogs are particularly suited for use as components of cytotoxic therapeutic agents of the invention which include gene fusion products and immunoconjugates. Cytotoxic therapeutic agents or immunotoxins according to the present invention may be used to selectively eliminate any cell type to which the RIP component is targeted by the specific binding capacity of the second component of the agent, and are suited for treatment of diseases where the elimination of a particular cell type is a goal, such as autoimmune disease, cancer and graft-versus-host disease.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LJ	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## IMMUNOTOXINS COMPRISING GELONIN AND AN ANTIBODY

FIELD OF THE INVENTION

This application is a continuation-in-part of co-pending U.S. Patent Application Serial No. 08/064,691 filed May 12, 1993, which is a continuation-in-part of co-pending U.S. Patent Application Serial No. 07/988,430 filed December 9, 1992, which is in turn a continuation-in-part of pending U.S. Patent Application Serial No. 07/901,707 filed June 19, 1992, which in turn is a continuation-in-part of U.S. Patent Application Serial No. 07/787,567 filed November 4, 1991, now abandoned.

The present invention generally relates to materials useful as components of cytotoxic therapeutic agents. More particularly, the invention relates to ribosome-inactivating proteins, to analogs of ribosome-inactivating proteins, to polynucleotides encoding such proteins and analogs, some of which are specifically modified for conjugation to targeting molecules, and to gene fusions of polynucleotides encoding ribosome-inactivating proteins to polynucleotides encoding targeting molecules.

BACKGROUND

Ribosome-inactivating proteins (RIPs) comprise a class of proteins which is ubiquitous in higher plants. However, such proteins have also been isolated from bacteria. RIPs are potent inhibitors of eukaryotic protein synthesis. The N-glycosidic bond of a specific adenine base is hydrolytically cleaved by RIPs in a highly conserved loop region of the 28S rRNA of eukaryotic ribosomes thereby inactivating translation.

Plant RIPs have been divided into two types. Stirpe et al., *FEBS Lett.*, 195(1,2):1-8 (1986). Type I proteins each consist of a single peptide chain having ribosome-inactivating activity, while Type II proteins each consist of an A-chain, essentially equivalent to a Type I protein, disulfide-linked to a B-chain having cell-binding

-2-

properties. Gelonin, dodecandrin, tricosanthin, tricokirin, bryodin, *Mirabilis* antiviral protein (MAP), barley ribosome-inactivating protein (BRIP), pokeweed antiviral proteins (PAPs), saporins, luffins, and momordins are examples of Type I RIPs; whereas Ricin and abrin are examples of Type II RIPs.

Amino acid sequence information is reported for various ribosome-inactivating proteins. It appears that at least the tertiary structure of RIP active sites is conserved among Type I RIPs, bacterial RIPs, and A-chains of Type II RIPs. In many cases, primary structure homology is also found. Ready et al., *J. Biol. Chem.*, 259(24):15252-15256 (1984) and other reports suggest that the two types of RIPs are evolutionarily related.

Type I plant ribosome-inactivating proteins may be particularly suited for use as components of cytotoxic therapeutic agents. A RIP may be conjugated to a targeting agent which will deliver the RIP to a particular cell type in vivo in order to selectively kill those cells. Typically, the targeting agent (e.g., an antibody) is linked to the toxin by a disulfide bond which is reduced in vivo allowing the protein toxin to separate from the delivery antibody and become active intracellularly. Another strategy for producing targeted cytotoxic proteins is to express a gene encoding a cytotoxic protein fused to a gene encoding a targeting moiety. The resulting protein product is composed of one or more polypeptides containing the cytotoxic protein linked to, for example, at least one chain of an antibody.

A variety of such gene fusions are discussed in Pastan et al., *Science*, 254:1173-1177 (1991). However, these fusion proteins have been constructed with sequences from diphtheria toxin or *Pseudomonas aeruginosa* exotoxin A, both of which are ADP-ribosyltransferases of bacterial origin. These protein toxins are reported to intoxicate cells and inhibit protein synthesis by mechanisms which differ from those of the RIPs. Moreover, diphtheria toxin



-3-

and exotoxin A are structurally different from, and show little amino acid sequence similarity with, RIPs. In general, fusion proteins made with diphtheria toxin or exotoxin A have been immunogenic and toxic in animals, and are produced intracellularly in relatively low yield. Another strategy for producing a cytotoxic agent is to express a gene encoding a RIP fused to a gene encoding a targeting moiety. The resulting protein product is a single polypeptide containing a RIP linked to, for example, at least one chain of an antibody.

Because some RIPs, such as the Type I RIP gelonin, are primarily available from scarce plant materials, it is desirable to clone the genes encoding the RIPs to enable recombinant production of the proteins. It is also desirable to develop analogs of the natural proteins which may be easily conjugated to targeting molecules while retaining their natural biological activity because most Type I RIPs have no natural sites (i.e. available cysteine residues) for conjugation to targeting agents. Alternatively, it is desirable to develop gene fusion products including Type I RIPs as a toxic moiety and antibody substances as a targeting moiety.

The present invention also provides novel humanized or human-engineered antibodies and methods for producing such antibodies which may be conjugated or fused to various toxins. Such conjugations or fusions are useful in the treatment of various disease states, including autoimmune diseases and cancer.

There are several reports relating to replacement of amino acids in a mouse antibody with amino acids normally occurring at the analogous position in the human form of the antibody. See, e.g., Junghaus, et al., *Cancer Res.*, 50: 1495-1502 (1990) and other publications which describe genetically-engineered mouse/human chimeric antibodies. Also by genetic engineering techniques, the genetic information from murine hypervariable complementarity determining regions (hereinafter referred

-4-

to as "CDRs") may be inserted in place of the DNA encoding the CDRs of a human monoclonal antibody to generate a construct encoding a human antibody with murine CDRs. See, e.g., Jones, et al., *Nature*, 321: 522-525 (1986).

5 Protein structure analysis on such "CDR-grafted" antibodies may be used to "add back" murine residues in order to restore lost antigen-binding capability, as described in Queen, et al, *Proc. Natl. Acad. Sci. (USA)*, 86:10029-10033 (1989); Co, et al., *Proc. Nat. Acad. Sci. (USA)*, 88: 2869-2873 (1991). However, a frequent result of CDR-grafting is that the specific binding activity of the resulting humanized antibodies may be diminished or completely abolished.

15 As demonstrated by the foregoing, there exists a need in the art for cloned genes encoding Type I RIPs, for analogs of Type I RIPs which may be easily conjugated to targeting molecules, and for gene fusion products comprising Type I RIPs, and especially wherein such gene fusions also comprise an humanized antibody portion.

#### 20 SUMMARY OF THE INVENTION

The present invention provides purified and isolated polynucleotides encoding Type I RIPs, Type I RIPs having a cysteine available for disulfide bonding to targeting molecules and fusion products comprising Type I RIPs. Vectors comprising the polynucleotides and host cells transformed with the vectors are also provided.

25 A purified and isolated polynucleotide encoding natural sequence gelonin (SEQ ID NO: 11), and a host cell including a vector encoding gelonin of the type deposited as ATCC Accession No. 68721 are provided. Further provided are a purified and isolated polynucleotide encoding natural sequence barley ribosome-inactivating protein and a purified and isolated polynucleotide encoding momordin II.

35 Some of the polynucleotides mentioned above encode fusion proteins of the present invention comprising gelonin (SEQ ID NO: 2) or another RIP and an antibody or a

-5-

fragment comprising an antigen-binding portion thereof. Several alternate forms of fusion proteins comprising gelonin are contemplated herein. For example, the fusion protein may contain a single RIP fused to a monovalent antibody binding moiety, such as a Fab or single chain antibody. Alternatively, multivalent forms of the fusion proteins may be made and may have greater activity than the monovalent forms. In preferred embodiments of the invention, gelonin may be fused to either the carboxy or the amino terminus of the antibody or antigen-binding portion of thereof. Also in a preferred embodiment of the invention, the antibody or fragment thereof comprising an antigen-binding portion may be an he3 antibody, an he3-Fab, an he3 Fd, single-chain antibody, or an he3 kappa fragment. The antibody or antigen-binding portion thereof may be fused to gelonin by means of a linker peptide, preferably a peptide segment of shiga-like toxin as shown in SEQ ID NO: 56 or a peptide segment of Rabbit muscle aldolase as shown in SEQ ID NO: 57 or a human muscle aldolase, an example of which is reported in Izzo, et al., *Eur. J. Biochem*, 174: 569-578 (1988), incorporated by reference herein.

Analogous of a Type I plant RIP are defined herein as non-naturally occurring polypeptides that share the ribosome-inactivating activity of the natural protein but that differ in amino acid sequence from the natural type I RIP protein in some degree but less than they differ from the amino acid sequences of other Type I plant RIP. Preferred analogs according to the present invention are analogous of Type I plant RIPs each having a cysteine available for disulfide bonding located at a position in its amino acid sequence from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. SEQ ID NO: 1 represents the amino acid sequence of ricin A-chain. Other preferred analogs according to the invention are Type I RIPs each having a cysteine available for disulfide bonding at a position in

-6-

the analog that is on the surface of the protein in its natural conformation and that does not impair native folding or biological activity of the ribosome-inactivating protein. Analogs of bacterial RIPs are also contemplated  
5 by the present invention.

The present invention provides an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not  
10 naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog corresponding to position 259 in SEQ ID NO: 1 or at a position in the amino acid sequence in the analog  
15 corresponding to a position from position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

An analog according to the present invention may be an analog of gelonin. In an analog of gelonin according to the present invention, the cysteine may be at a position  
20 in the analog from position 244 to the carboxyl terminal position of the analog, more preferably at a position in the analog from position 247 to the carboxyl terminal position of the analog, and most preferably at position 244, at position 247, or at position 248 of the amino acid  
25 sequence of the analog. In these analogs, it is preferred that the gelonin cysteine residues at positions 44 and 50 be replaced with non-cysteine residues such as alanine.

An analog according to the present invention may be an analog of barley ribosome-inactivating protein. Preferably, a cysteine in such an analog is at a position  
30 in the analog from position 256 to the carboxyl terminal position, and more preferably the cysteine is at a position in the amino acid sequence of the analog from position 260 to the carboxyl terminal position of the analog. Most  
35 preferably, in these regions, the cysteine is at position 256, at position 270, or at position 277 of the amino acid sequence of the analog.

-7-

An analog according to the present invention may be an analog of momordin II.

5       Analogues according to the present invention may have a cysteine in the amino acid sequence of the analog at a position which corresponds to a position within one amino acid of position 259 of SEQ ID NO: 1. Such an analog may be an analog of gelonin, of barley ribosome-inactivating protein, or of momordin II.

10       The present invention also provides a polynucleotide encoding an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type  
15       I ribosome-inactivating protein, and which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. The polynucleotide may encode an analog of  
20       gelonin, preferably an analog wherein the cysteine is at a position in the amino acid sequence of the analog from position 244 to the carboxyl terminal position of the analog, more preferably wherein the cysteine is at a position in the analog from position 247 to the carboxyl  
25       terminal position of the analog, and most preferably the cysteine is at position 244, at position 247 or at position 248 of the amino acid sequence of the analog. It is preferred that a polynucleotide according to the present invention encode a gelonin analog wherein the native  
30       gelonin cysteine residues at positions 44 and 50 are replaced with non-cysteine residues, such as alanine.

35       A polynucleotide according to the present invention may encode an analog of barley ribosome-inactivating protein, preferably an analog wherein the cysteine is at a position in the analog from position 256 to the carboxyl terminal position of the analog, more preferably wherein the cysteine is at a position in the

-8-

analog from position 260 to the carboxyl terminal position of the analog, and most preferably wherein the cysteine is ~~at position 256, at position 270 or at position 277~~ of the amino acid sequence of the analog.

5           A polynucleotide according to the present invention may encode an analog of mormordin II.

          The present invention provides a vector including a polynucleotide encoding an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at a amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

          The present invention further provides a host cell including a DNA vector encoding an analog of a Type I ribosome-inactivating protein, which analog has a cysteine available for intermolecular disulfide bonding at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. In such a host cell the vector may encode an analog of gelonin, especially an analog wherein the cysteine is at position 247 of the amino acid sequence of the analog, such as in the host cell deposited as ATCC Accession No. 69009.

          A host cell according to the present invention may include a vector encoding barley ribosome-inactivating protein, especially preferred is a host cell containing a BRIP analog wherein the cysteine is at position 277, such as in the host cell deposited on October 2, 1991 with the

-9-

American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 as ATCC Accession No. 68722. Particularly preferred are prokaryotic host cells because such cells may be less sensitive to the action of RIPs as compared to eukaryotic cells.

The present invention also provides an agent toxic to a cell including an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to the cell, which cysteine is at an amino acid position in the analog corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and which cysteine is located in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog. The agent may include an analog of gelonin, preferably an analog wherein the cysteine is at a position in the analog from position 247 to the carboxyl terminal position of the analog, and more preferably wherein the cysteine is at position 247 or 248 of the amino acid sequence of analog. An agent including an analog wherein the native gelonin cysteine residues at positions 44 and 50 are replaced with non-cysteine residues, such as alanine is preferred.

An agent according to the present invention may include an analog of barley ribosome-inactivating protein, preferably an analog wherein the cysteine is at a position in the analog from position 260 to the carboxyl terminal position of the analog, more preferably wherein the cysteine is at a position in the analog from position 270 to the carboxyl terminal position of the analog, and most preferably wherein the cysteine is at position 256, at position 270 or at position 277 of the amino acid sequence of the analog.

An agent according to the present invention may include an analog of momordin II.

-10-

The present invention provides an agent wherein the Type I ribosome-inactivating protein is linked to an antibody, particularly to an H65 antibody or to an antibody fragment, more particularly to an antibody fragment selected from the group consisting of chimeric and human engineered antibody fragments, and most particularly to a Fab antibody fragment, a Fab' antibody fragment or a F(ab')<sub>2</sub> antibody fragment. It is highly preferred that an agent according to the present invention include a chimeric or human engineered antibody fragment selected from the group consisting of a Fab antibody fragment, a Fab' antibody fragment and a F(ab')<sub>2</sub> antibody fragment.

A method according to the present invention for preparing an analog of a Type I ribosome-inactivating protein includes the step of expressing in a suitable host cell a polynucleotide encoding a Type I ribosome-inactivating fusion protein or type I RIP (especially gelonin) having a cysteine available for intermolecular disulfide bonding substituted (e.g., by site-directed mutagenesis of the natural DNA sequence encoding the RIP or by chemical synthesis of a DNA sequence encoding the RIP analog) at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein, which cysteine is located at a position in the amino acid sequence of the analog from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of the analog.

A product according to the present invention may be a product of a method including the step of expressing in a suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein, which cysteine is located at a position in the amino acid sequence of the



-11-

analog from the position corresponding to position 251 in  
SEQ ID NO: 1 to the carboxyl terminal position of the  
analog.

5 The present invention provides a method for  
preparing an agent toxic to a cell including the step of  
linking an analog of a Type I ribosome-inactivating protein  
through a cysteine to a molecule which specifically binds  
to the cell, which analog has the cysteine at an amino acid  
10 position corresponding to a position not naturally  
available for intermolecular disulfide bonding in the Type  
I ribosome-inactivating protein and which cysteine is  
located at a position in the amino acid sequence of the  
analog from the position corresponding to position 251 in  
SEQ ID NO: 1 to the carboxyl terminal position of the  
15 analog.

According to the present invention, a method for  
treating a disease in which elimination of particular cells  
is a goal may include the step of administering to a  
patient having the disease a therapeutically effective  
20 amount of an agent toxic to the cells including a type I  
RIP (especially gelonin fused to or an analog of a Type I  
ribosome-inactivating protein linked through a cysteine to  
a molecule which specifically binds to the cell, the analog  
having the cysteine at an amino acid position corresponding  
25 to a position not naturally available for intermolecular  
disulfide bonding in the Type I ribosome-inactivating  
protein and the cysteine being located at a position in the  
amino acid sequence of the analog from the position  
corresponding to position 251 in SEQ ID NO: 1 to the  
30 carboxyl terminal position of the analog.

Useful target cells for immunotoxins of the  
present invention include, but are not limited to, cells  
which are pathogenic, such as cancer cells, autoimmune  
cells, and virally-infected cells. Such pathogenic cells  
35 may be targeted by antibodies or other targeting agents of  
the invention which are joined, either by genetic  
engineering techniques or by chemical cross-linking, to an

-12-

RIP. Specifically useful targets include tumor-associated antigens (e.g., on cancer cells), cell differentiation markers (e.g., on autoimmune cells), parasite-specific antigens, bacteria-specific antigens, and virus-specific antigens.

5 The present invention also provides an analog of a Type I ribosome-inactivating protein, wherein the analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein the analog retains the ribosome-inactivating activity of the Type I ribosome-inactivating protein.

10 Such a fusion protein or an analog may be a fusion protein or an analog wherein the Type I ribosome inactivating protein is gelonin, and the analog is preferably an analog of gelonin wherein the cysteine is at position 10 of the amino acid sequence of the analog as encoded in a vector in a host cell deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 as ATCC Accession No. 69008 on June 9, 1992. Other such gelonin analogs include those wherein the cysteine is at a position 60, 103, 146, 184 or 215 in the amino acid sequence of the gelonin analog. It is preferred that the gelonin cysteine residues at positions 44 and 50 be replaced with non-cysteine residues such as alanine in these analogs.

20 The present invention further provides an analog of a Type I ribosome-inactivating protein wherein the analog includes only a single cysteine. Such an analog may be an analog of gelonin and is preferably an analog wherein the single cysteine is at position 10, position 44, position 50 or position 247 in the amino acid sequence of the analog, but the cysteine may be located at other positions defined by the invention as well.

-13-

The present invention provides a polynucleotide encoding an analog of a Type I ribosome-inactivating protein, wherein the analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein the analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

According to the present invention, a method for preparing an analog of a Type I ribosome-inactivating protein may include the step of expressing in suitable host cell a polynucleotide encoding a Type I ribosome-inactivating protein having a cysteine available for intermolecular disulfide bonding substituted at an amino acid position corresponding to a position not naturally available for disulfide bonding in the Type I ribosome-inactivating protein, the cysteine is located at a position corresponding to an amino acid position on the surface of ricin A-chain in its natural conformation and which analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

The present invention provides an agent toxic to a cell including an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to the cell, wherein the analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and wherein the analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

-14-

A method according to the present invention for preparing an agent toxic to a cell may include the step of linking an analog of a Type I ribosome-inactivating protein through a cysteine to a molecule which specifically binds to the cell, which analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and which analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

A method according to the present invention for treating a disease in which elimination of particular cells is a goal includes the step of administering to a patient having the disease a therapeutically effective amount of an agent toxic to the cells wherein the agent includes a type I RIP fused to or an analog of a Type I ribosome-inactivating protein linked by a disulfide bond through a cysteine to a molecule which specifically binds to the cell, which analog has a cysteine available for intermolecular disulfide bonding located at an amino acid position corresponding to a position not naturally available for intermolecular disulfide bonding in the Type I ribosome-inactivating protein and corresponding to a position on the surface of ricin A-chain in its natural conformation, and which analog retains ribosome-inactivating activity of the Type I ribosome-inactivating protein.

The RIP analogs of the invention are particularly suited for use as components of cytotoxic therapeutic agents. Cytotoxic agents according to the present invention may be used *in vivo* to selectively eliminate any cell type to which the RIP component is targeted by the specific binding capacity of the second component. To form cytotoxic agents, RIP analogs may be conjugated to

-15-

monoclonal antibodies, including chimeric and CDR-grafted antibodies, and antibody domains/fragments (e.g., Fab, Fab', F(ab')<sub>2</sub>, single chain antibodies, and Fv or single variable domains). Analogs of RIPs conjugated to monoclonal antibodies genetically engineered to include free cysteine residues are also within the scope of the present invention. Examples of Fab' and F(ab')<sub>2</sub> fragments useful in the present invention are described in co-pending, co-owned U.S. Patent Application Serial No. 07/714,175, filed June 14, 1991 and in International Publication No. WO 89/00999 published on February 9, 1989, which are incorporated by reference herein.

The RIP analogs of the invention may preferably be conjugated or fused to humanized or human engineered antibodies, such as the he3 antibody described herein. Such humanized antibodies may be constructed from mouse antibody variable domains, such as the mouse antibody H65 (SEQ ID NOS: 123 and 124). Specifically RIP analogs according to the present invention may be conjugated or fused to he3 human-engineered antibody light and heavy chain variable regions (SEQ ID NO: 125 and 126, respectively) or fragments thereof. A cell line producing an intact he3 immunoglobulin was deposited as ATCC Accession No. HB11206 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852.

RIPs according to the present invention may also be conjugated to targeting agents other than antibodies, for example, lectins which bind to cells having particular surface carbohydrates, hormones, lymphokines, growth factors or other polypeptides which bind specifically to cells having particular receptors. Immunoconjugates including RIPs may be described as immunotoxins. An immunotoxin may also consist of a fusion protein rather than an immunoconjugate.

The present invention provides gene fusions of an antigen-binding portion of an antibody (e.g., an antibody

-16-

light chain or truncated heavy chain, or a single chain antibody) or any targeting agent listed in the foregoing paragraph, linked to a Type I RIP. Preferably, the antigen-binding portion of an antibody or fragment thereof comprises a single chain antibody, a Fab fragment, or a F(ab')<sub>2</sub> fragment. Active antibodies generally have a conserved three-dimensional folding pattern and it is expected that any antibody which maintains that folding pattern will retain binding specificity. Such antibodies are expected to retain target enzymatic activity when incorporated into a fusion protein according to the present invention.

It is sometimes necessary that immunotoxins comprising cytotoxic components, such as RIPs, be attached to targeting agents via cleavable linkers (i.e., disulfides, acid-sensitive linkers, and the like) in order to allow intracellular release of the cytotoxic component. Such intracellular release allows the cytotoxic component to function unhindered by possible negative kinetic or steric effects of the attached antibody. Accordingly, gene fusions of the present invention may comprise a RIP gene fused, via a DNA segment encoding a linker protein as described above, to either the 5' or the 3' end of a gene encoding an antibody. If a linker is used, it preferably encodes a polypeptide which contains two cysteine residues participating in a disulfide bond and forming a loop which includes a protease-sensitive amino acid sequence (e.g., a segment of *E. coli* shiga-like toxin as in SEQ ID NO: 56) or a segment which contains several cathepsin cleavage sites (e.g., a segment of rabbit muscle aldolase as in SEQ ID NO: 57; a segment of human muscle aldolase; or a synthetic peptide including a cathepsin cleavage sites such as in SEQ ID NOs: 141 or 142). A linker comprising cathepsin cleavage sites as exemplified herein comprises the C-terminal 20 amino acids of RMA. However, that sequence differs by only one amino acid from human muscle aldolase and it is contemplated that muscle aldolase from human or

-17-

other sources may be used as a linker in the manner described below. The Type I RIP portion of the fused genes preferably encodes gelonin, BRIP or momordin II. Also preferably, the antibody portion of the fused genes comprises sequences encoding one of the chains of an antibody Fab fragment (i.e., kappa or Fd) and the fused gene is co-expressed in a host cell with the other Fab gene, or the antibody portion comprises sequences encoding a single chain antibody.

Alternatively, since fusion proteins of the present invention may be of low (approximately 55 kDa) molecular weight while maintaining full enzymatic activity, such fusions may be constructed without a linker and still possess cytotoxic activity. Such low-molecular weight fusions are not as susceptible to kinetic and steric hinderance as are the larger fusions, such as fusions involving IgG molecules. Therefore, cleavage of the RIP away from the fusion may not be necessary to facilitate activity of the RIP.

The present invention also provides a method for purifying a protein or immunotoxin comprising a ribosome-inactivating protein and a portion of an antibody including the steps of passing a solution containing the protein through an anion exchange column; applying the flow-through to a protein G column; and eluting the protein from the protein G column. The method may further comprise the steps of introducing the flow-through of the anion exchange column into a cation exchange column; exposing the cation exchange column to an eluent effective to elute said protein; and then applying the eluted protein to a protein G column, rather than applying the anion exchange column flow-through directly to a protein G column.

Immunotoxins according to the present invention, including immunoconjugates and fusion proteins (immunofusions), are suited for treatment of diseases where the elimination of a particular cell type is a goal, such as autoimmune disease, cancer, and graft-versus-host

-18-

disease. The immunotoxins are also suited for use in causing immunosuppression and in treatment of infections by ~~viruses such as the Human Immunodeficiency Virus.~~

Specifically illustrating polynucleotide sequences according to the present invention are the inserts in the plasmid pING3731 in *E. coli* MC1061 (designated strain G274) and in the plasmid pING3803 in *E. coli* E104 (designated strain G275), both deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, on October 2, 1991, and assigned ATCC Accession Nos. 68721 and 68722, respectively. Additional polynucleotide sequences illustrating the invention are the inserts in the plasmid pING3746 in *E. coli* E104 (designated strain G277) and in the plasmid pING3737 in *E. coli* E104 (designated strain G276), which were both deposited with the ATCC on June 9, 1992, and were respectively assigned Accession Nos. 69008 and 69009. Still other polynucleotide sequences illustrating the invention are the inserts in the plasmid pING3747 in *E. coli* E104 (designated strain G278), in the plasmid pING3754 in *E. coli* E104 (designated strain G279), in the plasmid pING3758 in *E. coli* E104 (designated strain G280) and in the plasmid pING3759 in *E. coli* E104 (designated strain G281), which plasmids were all deposited with the ATCC on October 27, 1992 and were assigned ATCC Accession Nos. 69101, 69102, 69103 and 69104, respectively.

As noted above, RIPs may preferably be conjugated or fused to humanized or human-engineered antibodies, such as he3. Thus, the present invention also provides novel proteins comprising an humanized antibody variable domain which is specifically reactive with an human CD5 cell differentiation marker. Specifically, the present invention provides proteins comprising the he3 light and heavy chain variable regions as shown in SEQ ID NOS: 95 or 96, respectively. DNA encoding certain he3 proteins is shown in SEQ ID NOS: 67 and 68.



-19-

In a preferred embodiment of the present invention, the protein comprising an humanized antibody variable region is an intact he3 immunoglobulin deposited as ATCC HB 11206.

5 Also in a preferred embodiment of the invention, the protein comprising an humanized antibody variable region is a Fab or F(ab')<sub>2</sub> or Fab fragment.

Proteins according to the present invention may be made by methods taught herein and in co-pending, co-  
10 owned U.S. patent application no. 07/808,464 by Studnicka et al. incorporated by reference herein; and modified antibody variable domains made by such methods may be used in therapeutic administration to humans either alone or as part of an immunoconjugate as taught in co-owned, co-  
15 pending U.S. Patent Application No. 07/787,567 by Better et al.

The present invention also provides methods for preparing a modified antibody variable domain useful in preparing immunotoxins and immunofusions by determining the  
20 amino acids of a subject antibody variable domain which may be modified without diminishing the native affinity of the domain for antigen while reducing its immunogenicity with respect to a heterologous species. As used herein, the term "subject antibody variable domain" refers to the  
25 antibody upon which determinations are made. The method includes the following steps: determining the amino acid sequence of a subject light chain and a subject heavy chain of a subject antibody variable domain to be modified; aligning by homology the subject light and heavy chains  
30 with a plurality of human light and heavy chain amino acid sequences; identifying the amino acids in the subject light and heavy chain sequences which are least likely to diminish the native affinity of the subject variable domain for antigen while, at the same time, reducing its  
35 immunogenicity by selecting each amino acid which is not in an interface region of the subject antibody variable domain and which is not in a complementarity-determining region or

-20-

in an antigen-binding region of the subject antibody variable domain, but which amino acid is in a position exposed to a solvent containing the antibody; changing each residue identified above which aligns with a highly or a moderately conserved residue in the plurality of human light and heavy chain amino acid sequences if said identified amino acid is different from the amino acid in the plurality.

Another group of sequences, such as those in Figures 1A and 1B may be used to determine an alignment from which the skilled artisan may determine appropriate changes to make.

The present invention provides a further method wherein the plurality of human light and heavy chain amino acid sequences is selected from the human consensus sequences in Figures 10A and 10B.

In general, human engineering according to the above methods may be used to treat various diseases against which monoclonal antibodies generally may be effective. However, humanized antibodies possess the additional advantage of reducing the immunogenic response in the treated patient.

Additional aspects and applications of the present invention will become apparent to the skilled artisan upon consideration of the detailed description of the invention which follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (RTA) (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein gelonin (SEQ ID NO: 2), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

FIG. 2 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-

-21-

inactivating protein BRIP (SEQ ID NO: 3), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

5       FIG. 3 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein momordin II (MOMOII) (SEQ ID NO: 4), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

10       FIG. 4 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein luffin (SEQ ID NO: 5), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

15       FIG. 5 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein atrichosanthin (TRICHO) (SEQ ID NO: 6), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

20       FIG. 6 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein momordin I (MOMOI) (SEQ ID NO: 7), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

25       FIG. 7 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein *Mirabilis* anti-viral protein (MAP) (SEQ ID NO: 8), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

30       FIG. 8 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-

-22-

inactivating protein pokeweed antiviral protein from seeds (PAPS) (SEQ ID NO: 9), wherein starred positions indicate amino acids invariant among the ricin A-chain and the Type I RIPs;

5                   FIG. 9 is a computer-generated alignment of the amino acid sequence of the ricin A-chain (SEQ ID NO: 1) with the amino acid sequence of the Type I ribosome-inactivating protein saporin 6 (SAP6) (SEQ ID NO: 10), wherein starred positions indicate amino acids invariant  
10 among the ricin A-chain and the Type I RIPs;

                  FIGS. 10A and 10B are alignments of the consensus amino acid sequences for the subgroups of light [hK1 (human kappa light chain subgroup 1), hK3 (human kappa light chain subgroup 3), hK2 (human kappa light chain subgroup 2), hL1  
15 (human lambda light chain subgroup 1), hL2 (human lambda light chain subgroup 2), hL3 (human lambda light chain subgroup 3), hL6 (human lambda light chain subgroup 6), hK4 (human kappa light chain subgroup 4), hL4 (human lambda light chain subgroup 4) and hL5 (human lambda light chain  
20 subgroup 5] and heavy chains [hH3 (human heavy chain subgroup 3), hH1 (human heavy chain subgroup 1) and hH2 (human heavy chain subgroup 2)], respectively, of human antibody variable domains;

                  FIG. 11 sets out the nucleotide sequences of the  
25 oligonucleotides utilized in the construction of the genes encoding modified V/J-regions of the light and heavy chains of the H65 mouse monoclonal antibody variable domain; and

                  FIGS. 12A and 12B are alignments of human light chain consensus hK1 and heavy chain consensus hH1 with the  
30 light and heavy chain sequences, respectively, of the variable domain of human antibody EU, human antibody TAC, human antibody TAC modified according to the present invention (prop) and human antibody TAC modified according to a different method (Que).

-23-

DETAILED DESCRIPTION

Nucleotide sequences of genes encoding three plant Type I RIPs and expression vectors containing the genes are provided by the present invention. A first plant RIP, gelonin, is produced by seeds of *Gelonium multiflorum*, a plant of the Euphorbiaceae family native to the tropical forests of eastern Asia, while a second plant RIP, BRIP, is synthesized by the common cereal grain barley. Momordin II, a third plant RIP, is produced in *Momordica balsamina* seeds. Analogs of BRIP are also provided by the present invention. The analogs were genetically engineered to include a cysteine free to participate in a intermolecular disulfide bond and were conjugated to antibody molecules without non-specific chemical derivatization of the RIP with crosslinking agents.

Type I RIP analogs of the present invention offer distinct advantages over the natural proteins for use as components of immunotoxins. Chemical treatment to introduce free sulfhydryl groups in the natural proteins lacking free cysteines typically involves the non-selective modification of amino acid side chains. This non-selectivity often results in antibodies conjugated to different sites on different RIP molecules (i.e., a heterogeneous population of conjugates) and also in a decrease in RIP activity if antibodies are conjugated in or near important regions of the RIP (e.g., the active site or regions involved in translocation across cell membranes). In contrast, RIP analogs according to the present invention may be conjugated to a single antibody through a disulfide bond to a specific residue of the analog resulting in reduced batch to batch variation of the immunoconjugates and, in some cases, immunoconjugates with enhanced properties (e.g., greater cytotoxicity or solubility).

Type I plant RIPs, as well as bacterial RIPs such as shiga and shiga-like toxin A-chains, are homologous to the ricin A-chain and are useful in the present invention.

-24-

Type I RIPs may be defined and sites for substitution of a cysteine in a RIP may be identified by ~~comparing the primary amino acid sequence of the RIP to the~~ natural ricin A-chain amino acid sequence, the tertiary structure of which has been described in Katzin et al.,  
5 *Proteins*, 10:251-259 (1991), which is incorporated by reference herein.

Amino acid sequence alignment defines Type I RIPs in that the ricin A-chain and the Type I plant RIPs have  
10 nine invariant amino acids in common. Based on the ricin sequence the invariant amino acids are tyrosine<sub>21</sub>, arginine<sub>29</sub>, tyrosine<sub>80</sub>, tyrosine<sub>123</sub>, leucine<sub>144</sub>, glutamic acid<sub>177</sub>, alanine<sub>178</sub>, arginine<sub>180</sub>, and tryptophan<sub>211</sub>. The ricin A-chain may be used as a model for the three-dimensional  
15 structure of Type I RIPs. A protein lacking a cysteine available for conjugation while having ribosome-inactivating activity and having the nine invariant amino acids when its primary sequence is compared to the primary sequence of the ricin A-chain [according to the alignment  
20 algorithm of Myers et al., *CABIOS COMMUNICATIONS*, 4(1):11-17 (1988), implemented by the PC/GENE program PALIGN (Intelligenetics, Inc., Mountain View, California) and utilizing the Dayhoff Mutation Data Matrix (MDM-78) as  
25 described in Schwartz et al., pp. 353-358 in *Atlas of Protein Sequence and Structure*, 5 Supp. 3, National Biomedical Research Foundation, Washington, D.C. (1978)] is defined as a Type I RIP herein and is expected to be useful  
in the present invention. "Corresponding" refers herein to amino acid positions which align when two amino acid  
30 sequences are compared by the strategy of Myers et al., *supra*.

The primary amino acid sequences of the Type I RIPs: gelonin, BRIP, momordin II, luffin [see Islam et al., *Agricultural Biological Chem.*, 54(5):1343-1345 (199)],  
35 atrichosanthin [see Chow et al., *J. Biol. Chem.*, 265:8670-8674 (1990)], momordin I [see Ho et al., *BBA*, 1088:311-314 (1991)], Mirabilis anti-viral protein [see Habuka et al.,

-25-

J. Biol. Chem., 264(12):6629-6637 (1989)], pokeweed antiviral protein isolated from seeds [see Kung et al., Agric. Biol. Chem., 54(12):3301-3318 (1990)] and saporin [see Benatti et al., Eur. J. Biochem., 183:465-470 (1989)] are individually aligned with the primary sequence of the ricin A-chain [see Halling et al., Nucleic Acids Res., 13:8019-8033 (1985)] in FIGs 1-9, respectively, according to the algorithm of Myers et al., *supra*, as specified above.

FIGs. 1-9 may be utilized to predict the amino acid positions of the Type I RIPs where cysteine residues may be substituted. Preferred amino acids for cysteine substitution are on the surface of the molecule and include any solvent accessible amino acids which will not interfere with proper folding of the protein if replaced with a cysteine. A region of the ricin A-chain comprising such amino acids is the carboxyl terminal region. Amino acids that should be avoided for replacement are those critical for proper protein folding, such as proline, and those that are solvent inaccessible. Also to be avoided are the nine amino acids invariant among RIPs, and the amino acids in or near regions comprising the active site of ricin A-chain as depicted in Figure 6 of Katzin et al., *supra*.

Therefore, a preferred region of substitution for Type I RIPs is their carboxyl terminal region which is solvent accessible and corresponds to the carboxyl terminal region where Type II RIP A-chains and B-chains are naturally linked by a disulfide bond. As shown in the examples, a cysteine may be substituted in positions in the amino acid sequence of a Type I RIP from the position corresponding to position 251 in SEQ ID NO: 1 to the carboxyl terminal position of said Type I RIP, resulting in RIP analogs which retain enzymatic activity and gain disulfide cross-linking capability. One preferred cysteine substitution position is near the position which corresponds to the cysteine at position 259 in the ricin A-chain.

-26-

For purposes of the present invention, immunotoxins comprise a class of compounds of which toxin-  
~~antibody fusions and immunoeonjugates are~~ examples. Immunotoxins are particularly suited for use in treatment  
5 of human autoimmune diseases and in the treatment of diseases in which depletion of a particular cell type is a goal, such as cancer. For example, treatment of autoimmune diseases with immunotoxins is described in International  
Publication No. WO89/06968 published August 10, 1989, which  
10 is incorporated by reference herein.

In any treatment regimen, the immunotoxins may be administered to a patient either singly or in a cocktail containing two or more immunotoxins, other therapeutic agents, compositions, or the like, including, but not  
15 limited to, immunosuppressive agents, tolerance-inducing agents, potentiators and side-effect relieving agents. Particularly preferred are immunosuppressive agents useful in suppressing allergic reactions of a host. Preferred immunosuppressive agents include prednisone, prednisolone,  
20 DECADRON (Merck, Sharp & Dohme, West Point, Pennsylvania), cyclophosphamide, cyclosporine, 6-mercaptopurine, methotrexate, azathioprine and i.v. gamma globulin or their combination. Preferred potentiators include monensin, ammonium chloride, perhexiline, verapamil, amantadine and  
25 chloroquine. All of these agents are administered in generally-accepted efficacious dose ranges such as those disclosed in the *Physician's Desk Reference*, 41st Ed., Publisher Edward R. Barnhart, New Jersey (1987). Patent Cooperation Treaty (PCT) patent application WO 89/069767  
30 published on August 10, 1989, discloses administration of an immunotoxin as an immunosuppressive agent and is incorporated by reference herein.

Immunotoxins of the present invention may be formulated into either an injectable or topical  
35 preparation. Parenteral formulations are known and are suitable for use in the invention, preferably for intramuscular or intravenous administration. The



-27-

formulations containing therapeutically-effective amounts of immunotoxins are either sterile liquid solutions, liquid suspensions, or lyophilized versions, and optionally contain stabilizers or excipients. Lyophilized compositions are reconstituted with suitable diluents, e.g., water for injection, saline, 0.3% glycine and the like, at a level of about from 0.01 mg/kg of host body weight to 10 mg/kg where the biological activity is less than or equal to 20 ng/ml when measured in a reticulocyte lysate assay. Typically, the pharmaceutical compositions containing immunotoxins of the present invention are administered in a therapeutically effective dose in a range of from about 0.01 mg/kg to about 5 mg/kg of the patient. A preferred, therapeutically effective dose of the pharmaceutical composition containing immunotoxins of the invention is in a range of from about 0.01 mg/kg to about 0.5 mg/kg body weight of the patient administered over several days to two weeks by daily intravenous infusion, each given over a one hour period, in a sequential patient dose-escalation regimen.

Immunotoxin compositions according to the invention may be formulated into topical preparations for local therapy by including a therapeutically effective concentration of immunotoxin in a dermatological vehicle. The amount of immunotoxin to be administered, and the immunotoxin concentration in the topical formulations, depend upon the vehicle selected, the clinical condition of the patient, the systemic toxicity and the stability of the immunotoxin in the formulation. Thus, a physician knows to employ the appropriate preparation containing the appropriate concentration of immunotoxin in the formulation, as well as the appropriate amount of formulation to administer depending upon clinical experience with the patient in question or with similar patients. The concentration of immunotoxin for topical formulations is in the range of greater than from about 0.1 mg/ml to about 25 mg/ml. Typically, the concentration of

-28-

immunotoxin for topical formulations is in the range of greater than from about 1 mg/ml to about 20 mg/ml. Solid dispersions of immunotoxins according to the invention, as well as solubilized preparations, may be used. Thus, the  
5 precise concentration to be used in the vehicle is subject to modest experimental manipulation in order to optimize the therapeutic response. For example, greater than about 10 mg immunotoxin/100 grams of vehicle may be useful with  
10 1% w/w hydrogel vehicles in the treatment of skin inflammation. Suitable vehicles, in addition to gels, are oil-in-water or water-in-oil emulsions using mineral oils, petroleum and the like.

Immunotoxins according to the invention may be optionally administered topically by the use of a  
15 transdermal therapeutic system [Barry, *Dermatological Formulations*, p. 181 (1983) and literature cited therein]. While such topical delivery systems may be designed for transdermal administration of low molecular weight drugs, they are capable of percutaneous delivery. Further, such  
20 systems may be readily adapted to administration of immunotoxin or derivatives thereof and associated therapeutic proteins by appropriate selection of the rate-controlling microporous membrane.

Topical preparations of immunotoxin either for  
25 systemic or local delivery may be employed and may contain excipients as described above for parenteral administration and other excipients used in a topical preparation such as cosolvents, surfactants, oils, humectants, emollients, preservatives, stabilizers and antioxidants.  
30 Pharmacologically-acceptable buffers may be used, e.g., Tris or phosphate buffers. The topical formulations may also optionally include one or more agents variously termed enhancers, surfactants, accelerants, adsorption promoters or penetration enhancers, such as an agent for enhancing  
35 percutaneous penetration of the immunotoxin or other agents. Such agents should desirably possess some or all of the following features as would be known to the

-29-

ordinarily skilled artisan: pharmacological inertness, non-promotive of body fluid or electrolyte loss, compatible with immunotoxin (non-inactivating), and capable of formulation into creams, gels or other topical delivery systems as desired.

Immunotoxins according to the present invention may also be administered by aerosol to achieve localized delivery to the lungs. This is accomplished by preparing an aqueous aerosol, liposomal preparation or solid particles containing immunotoxin. Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of immunotoxin together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary depending upon the requirements for the particular immunotoxin, but typically include: nonionic surfactants (Tweens, Pluronic, or polyethylene glycol); innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin; amino acids such as glycine; and buffers, salts, sugars or sugar alcohols. The formulations may also include mucolytic agents as well as bronchodilating agents. The formulations are sterile. Aerosols generally are prepared from isotonic solutions. The particles optionally include normal lung surfactants.

Alternatively, immunotoxins of the invention may be administered orally by delivery systems such as proteinoid encapsulation as described by Steiner, et al., U.S. Patent No. 4,925,673, incorporated by reference herein. Typically, a therapeutically-effective oral dose of an immunotoxin according to the invention is in the range from about 0.05 mg/kg body weight to about 50 mg/kg body weight per day. A preferred effective dose is in the range from about 0.05 mg/kg body weight to about 5 mg/kg body weight per day.

Immunotoxins according to the present invention may be administered systemically, rather than topically, by injection intramuscularly, subcutaneously, intrathecally or

-30-

intraperitoneally or into vascular spaces, particularly into the joints, e.g., intraarticular injection at a dosage of greater than about 1  $\mu\text{g}/\text{cc}$  joint fluid/day. The dose will be dependent upon the properties of the specific immunotoxin employed, e.g., its activity and biological half-life, the concentration of immunotoxin in the formulation, the site and rate of dosage, the clinical tolerance of the patient involved, the disease afflicting the patient and the like, as is well within the skill of the physician.

The immunotoxins of the present invention may be administered in solution. The pH of the solution should be in the range of pH 5 to 9.5, preferably pH 6.5 to 7.5. The immunotoxin or derivatives thereof should be in a solution having a suitable pharmaceutically-acceptable buffer such as phosphate, Tris(hydroxymethyl)aminomethane-HCl or citrate and the like. Buffer concentrations should be in the range of 1 to 100 mM. The immunotoxin solution may also contain a salt, such as sodium chloride or potassium chloride in a concentration of 50 to 150 mM. An effective amount of a stabilizing agent such as an albumin, a globulin, a gelatin, a protamine or a salt of protamine may also be included, and may be added to a solution containing immunotoxin or to the composition from which the solution is prepared.

Systemic administration of immunotoxin may be made daily and is generally by intramuscular injection, although intravascular infusion is acceptable. Administration may also be intranasal or by other nonparenteral routes. Immunotoxins of the present invention may also be administered via microspheres, liposomes or other microparticulate delivery systems placed in certain tissues including blood. Topical preparations are applied daily directly to the skin or mucosa and are then preferably occluded, i.e., protected by overlaying a bandage, polyolefin film or other barrier impermeable to the topical preparation.

-31-

The following Examples are illustrative of practice of the invention but are not to be construed as limiting the invention. The present application is broadly organized as follows. The first portion of the application broadly teaches the preparation, expression and properties of an exemplary RIP, gelonin. A second portion of the application teaches the preparation of human-engineered antibodies. A third portion of the application teaches the construction and properties of immunoconjugates, comprising an RIP and an antibody or fragment thereof comprising an antigen-binding portion. A forth portion of the application relates to the preparation and properties of immunofusion proteins comprising an RIP and an antibody or fragment thereof comprising an antigen-binding portion. A fifth portion of the application teaches the preparation and properties of the RIP Barley ribosome-inactivating protein and a final aspect of the invention provides the preparation and properties of the RIP momordin.

Specifically, Example 1 relates to the preparation of the RIP gelonin. Construction of expression vector, comprising the gelonin gene, including expression and purification of gelonin, is taught in Example 2. The assembly of gelonin genes with cysteine residues available for conjugation is taught in Example 3 and Example 4 provides results of a reticulocyte lysate assay performed on gelonin.

Example 5 teaches the construction of human-engineered antibodies for use in immunotoxins of the invention and Example 6 demonstrates transfection of he3 genes, expression of those genes, and purification of the products thereof.

Example 7 next teaches the preparation of gelonin immunoconjugates. The procedures and results of whole cell kill assays are next presented in Example 8. Various properties of gelonin immunoconjugates are taught in Example 9 and Examples 10 and 11 teach the pharmacokinetics of two types of immunoconjugates. Examples 12 and 13 teach

-32-

the immunogenicity of immunoconjugates of the invention and the *in vivo* efficacy of those immunoconjugates, respectively.

5 The construction of genes encoding gelonin immunofusions is taught in Examples 14, 15, 16, 17 and 18. Example 19 teaches alternative cathepsin cleavable linkers for use in the immunofusions of the invention. The expression and purification of various genes encoding immunoconjugates are presented in Example 20 and their  
10 activity properties are presented in Example 21.

The construction of genes encoding the RIP, BRIP, and its expression and properties are taught in Examples 22, 23, and 24.

15 Finally, construction of genes encoding momordin and properties of momordin on expression are taught in Example 25.

### Example 1

#### Preparation Of Gelonin

20 The cloning of the gelonin gene according to the present invention obviates the requirement of purifying the RIP gene product from its relatively scarce natural source, *G. multiflorum* seeds. Cloning also allows development of gelonin analogs which may be conjugated to antibodies without prior chemical derivatization and also allows  
25 development of gelonin gene fusion products.

#### A. Preparation Of RNA From *G. Multiflorum* Seeds

Total RNA was prepared from *Gelonium* seeds (Dr. Michael Rosenblum, M.D. Anderson Cancer Center, Houston, Texas) by a modification of the procedure for preparation  
30 of plant RNA described in Ausubel et al., eds., *Current Protocols in Molecular Biology*, Wiley & Sons, 1989. Briefly, 4.0 grams of seeds were ground to a fine powder in a pre-cooled (-70°C) mortar and pestle with liquid N<sub>2</sub>. The powder was added to 25 ml Grinding buffer (0.18M Tris,  
35 0.09M LiCl, 4.5mM EDTA, 1% SDS, pH 8.2) along with 8.5 ml

-33-

of phenol equilibrated with TLE (0.2M Tris, 0.1M LiCl, 5mM EDTA pH8.2). The mixture was homogenized using a Polytron PT-1035 (#5 setting). 8.5 ml of chloroform was added, mixed and incubated at 50°C for 20 minutes. The mixture was centrifuged at 3000 g for 20 minutes in a rotor precooled to 4°C and the aqueous phase was transferred to a new tube. 8.5 ml of phenol was added followed by 8.5 ml of chloroform and the mixture was recentrifuged. This extraction was repeated 3 times. The RNA in the aqueous phase was then precipitated by adding 1/3 volume 8M LiCl, and incubated at 4°C for 16 hours. Next, the RNA was pelleted by centrifugation for 20 minutes at 4°C. The pellet was washed with 5 ml of 2M LiCl, recentrifuged and resuspended in 2 ml of water. The RNA was precipitated by addition of NaOAc to 0.3M and 2 volumes of ethanol. The RNA was stored in 70% ethanol at -70°C.

#### B. cDNA Preparation

cDNA was prepared from total *Gelonium* RNA by two methods. The first method involved making a cDNA library in the bacterial expression plasmid pCDNAII using the Librarian II cDNA Library Construction System kit (Invitrogen). Approximately 5 µg of total RNA was converted to first strand cDNA with a 1:1 mixture of random primers and oligo-dT. Second strand synthesis with DNA polymerase I was performed as described by the system manufacturer. Double stranded cDNA was ligated to *Bst*XI linkers and size fractionated. Pieces larger than about 500 bp were ligated into the expression vector provided in the kit. Individual vectors were introduced into *E. coli* either by transformation into high-efficiency competent cells or by electroporation into electrocompetent cells. Electroporation was performed with a BTX100 unit (BTX, San Diego, CA) in 0.56µ Flatpack cells as recommended by BTX based on the method of Dower et al., *Nucleic Acids Res.*, 16:6127-6145 (1988), at a voltage amplitude of 850 V and a

-34-

pulse length of 5 mS. The resulting library consisted of approximately 150,000 colonies.

5       The second method involved generating cDNA using the RNA-PCR kit sold by Perkin-Elmer-Cetus. About 100 ng of total Gelonium RNA was used as template for cDNA synthesis.

#### C. Determination Of The Gelonin Protein Sequence

10       The partial sequence of the native gelonin protein was determined by direct amino acid sequence analysis using automated Edman degradation as recommended by the manufacturer using an Applied Biosystems model 470A protein sequencer. Proteolytic peptide fragments of gelonin (isolated from the same batch of seeds as the total RNA) were sequenced.

#### 15       D. Cloning Of The Gelonin Gene

Three overlapping gelonin cDNA fragments were cloned and a composite gelonin gene was assembled from the three fragments.

##### 20       1. Cloning Of The Fragment Encoding The Middle Amino Acids Of Gelonin In Vector pING3823

25       Degenerate DNA primers based on the gelonin partial amino acid sequences were used to PCR-amplify segments of the cDNA generated with Perkin-Elmer-Cetus kit. Six primers were designed based on regions of the gelonin amino acid sequence where degeneracy of the primers could be minimized. Appropriate pairs of primers were tested for amplification of a gelonin gene fragment. Products of the expected DNA size were identified as ethidium bromide-stained DNA bands on agarose gels that DNA was treated with  
30       T4 DNA polymerase and then purified from an agarose gel. Only the primer pair consisting of primers designated gelo-7 and gelo-5 yielded a relatively pure product of the expected size. The sequences of degenerate primers gelo-7 and gelo-5 are set out below using IUPAC nucleotide  
35       symbols.



-35-

Gelo-7 (SEQ ID NO: 14)

5' TTYAARGAYGCNCCNGAYGCNGCNTAYGARGG 3'

Gelo-5 (SEQ ID NO: 15)

3' TTYTTYATRATRCANTGNCGNCANCTRGTYCA 5'

5     Primer gelo-7 corresponds to amino acids 87-97 of gelonin while primer gelo-5 corresponds to amino acids 226-236. The blunt-ended DNA fragment (corresponding to amino acids 87 to 236 of gelonin) generated with primers gelo-7 and gelo-5 was cloned into pUC18 (BRL, Gaithersburg, Maryland).

10    The DNA sequence of the insert was determined, and the deduced amino acid sequence based on the resulting DNA sequence matched the experimentally determined gelonin amino acid sequence. The clone containing this gelonin segment was denoted pING3726.

15             The insert of clone pING3726 was labeled with <sup>32</sup>P and used as a probe to screen the 150,000-member Gelonium cDNA library. Only one clone hybridized to the library plated in duplicate. This clone was purified from the library and its DNA sequence was determined. The clone

20    contains a fragment encoding 185 of the 270 amino acids of gelonin (residues 25-209) and is denoted pING3823.

## 2. Cloning Of The Fragment Encoding The N-Terminal Amino Acids Of Gelonin

Based on the sequence determined for the gelonin gene segment in pING3726, exact oligonucleotide primers were designed as PCR amplification primers to be used in conjunction with a degenerate primer to amplify a 5' gelonin gene fragment and with a nonspecific primer to amplify a 3' gelonin gene fragment. cDNA generated using

25             the Perkin-Elmer-Cetus RNA-PCR kit was amplified.

30             To amplify the 5'-end of the gelonin gene, PCR amplification with a degenerate primer gelo-1 and an exact primer gelo-10 was performed. The sequences of the primers are set out below.

-36-

Gelo-1 (SEQ ID NO: 16)

5' GGN<sup>y</sup>TNGAYACNGTNWSNTTYWSNACNAARGG 3'

Gelo-10 (SEQ ID NO: 17)

3' TGTCTGAACCCGTAACCTGGTAA 5'

5 Primer gelo-1 corresponds to amino acids 1-11 of the  
gelonin gene while primer gelo-10 corresponds to amino  
acids 126-133. The product from the reaction was re-  
amplified with gelo-1 (SEQ ID NO: 16) and gelo-11 (an exact  
primer comprising sequences encoding amino acids 119-125 of  
10 gelonin) to confer specificity to the reaction product.  
The sequence of primer gelo-11 is listed below.

Gelo-11 (SEQ ID NO: 18)

3' CACTCTCCGTATATCTCTCTGT 5'

Hybridization with an internal probe confirmed that the  
15 desired specific gelonin DNA fragment was amplified. That  
fragment was cloned into pUC18 and the vector generated was  
designated pING3727. The fragment was sequenced, revealing  
that the region of the fragment (the first 27 nucleotides)  
corresponding to part of the degenerate primer gelo-1 could  
20 not be translated to yield the amino acid sequence upon  
which primer gelo-1 was originally based. This was not  
unexpected considering the degeneracy of the primer. The  
fragment was reamplified from the Gelonium cDNA with exact  
primers gelo-11 (SEQ ID NO: 18) and gelo-5' (which extends  
25 upstream of the 5' end of the gelonin gene in addition to  
encoding the first 16 amino acids of gelonin). The  
sequence of primer gelo-5' is set out below.

Gelo-5' (SEQ ID NO: 19)

5' TCAACCCGGGCTAGATACCGTGTCAT

30 TCTCAACCAAAGGTGCCACTTATATTA 3'

The resulting DNA fragment encodes the first 125 amino  
acids of gelonin. While the majority of the sequence is  
identical to the natural gelonin gene, the first 32  
nucleotides of the DNA fragment may be different. For the  
35 purposes of this application this N-terminal fragment is  
referred to as fragment GEL1-125.

-37-

### 3. Cloning Of The Fragment Encoding The C-Terminal Amino Acids Of Gelonin

~~-----To amplify the 3'-end of the gelonin gene as well~~  
as 3' untranslated sequences, PCR amplification with exact  
primers gelo-9 and XE-dT was performed. The sequence of  
each of the primers is set out below.

Gelo-9 (SEQ ID NO: 20)

5' CTTCATTTTGGCGGCACGTATCC 3'

XE-dT (SEQ ID NO: 21)

3' TTTTTTTTTTTTTTTTTTTAG

GGTGCATTCTGAACGTCGGAGCTC 5'

Primer gelo-9 corresponds to amino acids 107-113 of  
gelonin. Primer XE-dT consists of a 3' oligo-dT portion  
and a 5' portion containing the restriction sites *Hind*III  
and *Xho*I, and will prime any poly A-containing cDNA. The  
reaction product was reamplified with exact primers gelo-8  
and XE. The sequences of primers gelo-8 and XE are set out  
below.

Gelo-8 (SEQ ID NO: 22)

5' CTCGCTGGAAGGTGAGAA 3'

XE (SEQ ID NO: 23)

3' AGGGTGCATTCTGAACGTCGGAGCTC 5'

Primer gelo-8 consists of sequences encoding amino acids  
115-120 of gelonin while the primer XE corresponds to the  
5' portion of the XE-dT primer which contains *Hind*III and  
*Xho*I restriction sites. Hybridization with internal probes  
confirmed that the desired gelonin gene fragment was  
amplified. That fragment was then cloned into *pUC18* by two  
different methods. First, it was cloned as a blunt-ended  
fragment into the *Sma*I site of *pUC18* (the resulting vector  
was designated *pING3728*) and, second, it was cloned as an  
*Eco*RI to *Hind*III fragment into *pUC18* (this vector was  
designated *pING3729*). Both vector inserts were sequenced.  
The insert of *pING3728* encodes amino acids 114-270 of  
gelonin, while the insert of *pING3729* encodes amino acids  
184-270 of gelonin plus other 3' sequences.

-38-

#### 4. Assembly Of The Overlapping Gelonin DNA Fragments Into A Composite Gelonin Gene

To reassemble the C-terminal two-thirds of the gelonin gene, vector pING3729 was cut with *SspI* (one *SspI* site is located within the vector and the second is located about 80 bp downstream of the termination codon of the insert in the vector) and an *XhoI* linker (8 bp, New England Biolabs) was ligated to the resulting free ends. The DNA was then cut with *XhoI* and *EcoRI*, and the 350 bp fragment generated, encoding amino acids 185-270 of gelonin, was isolated. This 350 bp fragment was ligated adjacent to a *NcoI* to *EcoRI* fragment from pING3823 encoding amino acids 37-185 of gelonin in a intermediate vector denoted pING3730, thus reassembling the terminal 87% of the gelonin gene (amino acids 37-270).

Next, fragment GEL1-125 was cut with *SmaI* and *NcoI*, resulting in a fragment encoding amino acids 1-36 of gelonin which was ligated along with the *NcoI* to *XhoI* fragment of pING3730 into the vector pIC100. [pIC100 is identical to pING1500 described in Better, et al., *Science*, 240:1041-1043 (1988), incorporated by reference herein], except that it lacks 37 bp upstream of the *pelB* leader sequence. The 37 bp were eliminated by digestion of pING1500 with *SphI* and *EcoRI*, treatment with T4 polymerase, and religation of the vector. This manipulation regenerated an *EcoRI* site in the vector while eliminating other undesirable restriction sites.] Before ligation, the vector pIC100 had previously been digested with *SstI*, treated with T4 polymerase, and cut with *XhoI*. The ligation generated a new vector containing a complete gelonin gene which was designated plasmid pING3731 and deposited with The American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on October 2, 1991 as Accession No. 68721. The complete DNA sequence of the gelonin gene is set out in SEQ ID NO: 11.

-39-

Example 2A. Construction Of Expression  
Vectors Containing The Gelonin Gene

5 A first *E. coli* expression vector was constructed  
containing the gelonin gene linked to the *Erwinia*  
10 *carotovora pelB* leader sequence, and to the *Salmonella*  
*typhimurium araB* promoter. A basic vector containing the  
araB promoter is described in co-owned U.S. Patent No.  
5,028,530 issued July 2, 1991 which is incorporated by  
reference herein. The vector containing the araB promoter  
was cut with *EcoRI* and *XhoI*. Two DNA fragments were then  
ligated in tandem immediately downstream of the promoter.  
The fragment ligated adjacent to the promoter was a 131 bp  
15 fragment derived from *SstI* digestion, T4 polymerase  
treatment and digestion with *EcoRI* of the pIC100 vector  
which includes the leader sequence of the *E. carotovora*  
*pelB* gene. The translated leader sequence is a signal for  
secretion of the respective protein through the cytoplasmic  
membrane. The fragment ligated downstream of the leader  
20 sequence was a *SmaI* to *XhoI* fragment from pING3731 which  
contains the complete gelonin gene. Thus, the expression  
vector contains the gelonin gene linked to the *pelB* leader  
sequence and the araB promoter. This plasmid is designated  
pING3733.

25 A second expression vector may be constructed  
that is identical to the first except that the gelonin gene  
sequences encoding the nineteen C-terminal amino acids of  
gelonin are not included. The cDNA sequence of the gelonin  
gene predicted a 19 residue C-terminal segment that was not  
30 detected in any peptide fragments generated for  
determination of the gelonin amino acid sequence. These 19  
amino acids may represent a peptide segment that is cleaved  
from the mature toxin post-translationally, i.e. that is  
not present in the native protein. A similar C-terminal  
35 amino acid segment was identified in the plant toxin  
 $\alpha$ -trichosanthin [Chow et al., *J. Biol. Chem.*, 265:8670-8674  
(1990)]. Therefore, the expression product without the C-  
terminal fragment is of interest.

-40-

For construction of a gelonin expression vector without the 19 C-terminal amino acids of gelonin, PCR was used to amplify and alter the 3'-end of the gene. pING3728 was amplified with primers gelo-14 and gelo-9 (SEQ ID NO: 20). The sequence of primer gelo-14 is set out below.

Gelo-14 (SEQ ID NO: 24)

5' TGATCTCGAGTACTATTTAGGATCTTTATCGACGA 3'

Primer gelo-14, which corresponds to gelonin amino acids 245-256, introduces a termination codon (underlined in the primer sequence) in the gelonin gene sequence which stops transcription of the gene before the sequences encoding the terminal 19 amino acids of the gelonin and also introduces a *XhoI* site immediately downstream of the termination codon. The PCR product was cut with *XhoI* and *EcoRI*, and the resulting 208 bp fragment encoding amino acids 185-251 of gelonin was purified from an agarose gel. This fragment was ligated adjacent to the *NcoI* to *EcoRI* fragment from pING3823 encoding amino acids 37-185 of gelonin to generate plasmid pING3732. A final expression vector, pING3734, containing a gelonin gene with an altered 3'-end was generated by substituting an *NcoI* to *XhoI* fragment encoding amino acids 37-251 of gelonin from pING3732 into pING3733.

#### B. Identification Of The Native Gelonin 5'-End

Inverse PCR was used to identify a cDNA clone encoding the 5'-end of the mature gelonin gene. 5 µg of total *G. multiflorum* RNA was converted to cDNA using the Superscript Plasmid System (BRL, Gaithersburg, Maryland) with Gelo-11 (SEQ ID NO: 18) as a primer. Gelonin cDNA was self-ligated to generate covalent circular DNA and the ligated DNA was amplified by PCR with oligonucleotides Gelo-9 (SEQ ID NO: 20) and Gelo-16. The sequence of primer Gelo-16 is set out below.

Gelo-16 (SEQ ID NO: 25)

5' GTAAGCAGCATCTGGAGCATCT 3'

The PCR product was size-fractionated on an agarose gel and DNAs larger than 300 bp were cloned into *SmaI* cut pUC18.

-41-

Several clones were sequenced with the primer Gelo-18, the sequence of which is set out below.

-----Gelo-18 (SEQ ID NO: 26)-----

5' CATTCAAGAAATTCACGTAGG 3'

5 A clone identified as having the largest gelonin-specific insert was designated pING3826. The DNA sequence of pING3826 included the first 32 nucleotides of the natural, mature gelonin gene not necessarily present in gelonin expression plasmids pING3733 and pING3734. The complete  
10 DNA sequence of the natural gelonin gene is set out in SEQ ID NO: 11.

C. Construction Of Expression Vectors.  
Containing A Gelonin Gene With A Natural 5' End

15 Derivatives of expression vectors pING3733 and pING3734 (described above) containing a gelonin gene with the natural 5' sequence were generated as follows. The 5'-end of gelonin was amplified from pING3826 with the PCR primers Gelo-16 (SEQ ID NO: 24) and Gelo-17, the sequence of which is set out below.

20 Gelo-17 (SEQ ID NO: 27)

5' GGCCTGGACACCGTGAGCTTTAG 3'

The 285 bp PCR product was treated with T4 polymerase and cut with NcoI. The resulting 100 bp 5'-end DNA fragment was isolated from an agarose gel and ligated adjacent to  
25 the 120 bp *pelB* leader fragment from pIC100 (cut with *Sst*I, treated with T4 polymerase and cut with *Pst*I) into either pING3733 or pING3734 digested with *Pst*I and *Nco*I. The resulting plasmids pING3824 and pING3825 contain the entire native gelonin gene and the native gelonin gene minus the  
30 nineteen amino acid carboxyl extension, respectively, linked to the *pelB* leader and under the transcriptional control of the *araB* promoter. The gene construct without the nineteen amino acid carboxyl extension in both pING3734 and pING3825 encodes a protein product referred to in this  
35 application as "recombinant gelonin".

-42-

#### D. Purification Of Recombinant Gelonin

Recombinant gelonin was purified by the following procedure:--~~E.-coli~~ fermentation broth was concentrated and buffer-exchanged to 10 mM sodium phosphate at pH 7.0 by using an S10Y10 cartridge over a DC10 unit (Amicon) the concentrated and buffer-exchanged material was applied to a CM52 column (100 g, 5X10 cm). The column was washed with 1 L of starting buffer and eluted with a 0 to 300 mM NaCl gradient in starting buffer (750 ml total volume). The pure gelonin containing fractions were pooled (elution was from 100-250 mM NaCl), concentrated over an Amicon YM10 membrane, equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and stored frozen at -20°C. A further purification step was attempted using Blue Toyopearl chromatography. However, this procedure did not result in an increased purity of material and resulted in an approximate 50% loss of the starting material.

#### Example 3

##### Assembly Of Gelonin Genes With Cysteine Residues Available For Conjugation

The wild-type gelonin protein has two cysteine residues at positions 44 and 50 which are linked by an endogenous disulfide bond. The protein contains no free cysteine residue directly available for conjugation to antibodies or other proteins. Analogs of gelonin which contain a free cysteine residue available for conjugation were generated by three different approaches. In one approach, various residues along the primary sequence of the gelonin were replaced with a cysteine residue, creating a series of analogs which contain an odd number of cysteine residues. In another approach, one of the two endogenous cysteines was replaced by alanine, creating a molecule which lacks an intrachain disulfide bond but contains a single, unpaired cysteine. In yet another approach both endogenous cysteines were replaced by alanines and a third non-cysteine residue was replaced by a cysteine, creating an analog with a single, unpaired cysteine.



-43-

Fifteen analogs of gelonin were constructed. Ten non-cysteine residues of gelonin were targeted for substitution with a cysteine residue. Comparison of the amino acid sequence of gelonin to the natural amino acid sequence and tertiary structure of the ricin A-chain (see FIG. 1) suggested that these positions would be at the surface of the molecule and available for conjugation. Each of the ten gelonin analogs include a cysteine substituted in place of one of the following residues: lysine<sub>10</sub>, asparagine<sub>60</sub>, isoleucine<sub>103</sub>, aspartic acid<sub>146</sub>, arginine<sub>184</sub>, serine<sub>215</sub>, asparagine<sub>239</sub>, lysine<sub>244</sub>, aspartic acid<sub>247</sub>, and lysine<sub>248</sub>, and the analogs have respectively been designated Gel<sub>C10</sub>, Gel<sub>C60</sub>, Gel<sub>C103</sub>, Gel<sub>C146</sub>, Gel<sub>C184</sub>, Gel<sub>C215</sub>, Gel<sub>C239</sub>, Gel<sub>C244</sub>, Gel<sub>C247</sub>, and Gel<sub>C248</sub>.

Two analogs of gelonin were constructed in which one of the native gelonin cysteines that participates in an endogenous disulfide bond was replaced with a non-cysteine residue. Specifically, the cysteine at position 50 was replaced with an alanine residue, creating a gelonin analog (designated Gel<sub>A50(C44)</sub>, shown in SEQ ID NO: 99) which has a cysteine available for disulfide bonding at position 44. The Gel<sub>A50(C44)</sub> analog has been referred to previously as Gel<sub>C44</sub> (see, e.g., co-owned, co-pending U.S. Patent Application Serial No. 07/988,430, incorporated by reference herein). Conversely, the cysteine at position 44 was replaced with an alanine residue, resulting in an analog (designated Gel<sub>A44(C50)</sub>, shown in SEQ ID NO: 100) which has a cysteine available for disulfide bonding at position 50. The Gel<sub>A44(C50)</sub> analog has been referred to previously as Gel<sub>C50</sub> (see, e.g., co-owned, co-pending U.S. Patent Application Serial No. 07/988,430, incorporated by reference herein). The combined series of the foregoing twelve analogs thus spans the entire length of the mature gelonin protein.

Another gelonin analog (Gel<sub>AAAA50</sub> SEQ ID NO: 101) was constructed in which both native gelonin cysteines were replaced with alanines. The Gel<sub>AAAA50</sub> analog has been referred to previously as Gel<sub>C44AC50A</sub> (see, e.g., co-owned, co-

-44-

pending U.S. Patent Application Serial No. 07/988,430, incorporated by reference herein). Two additional analogs were constructed which have alanine residues substituted in place of both native cysteines and have either a cysteine residue substituted in place of the native lysine at position 10 (Gel<sub>C10A44A50</sub>, shown in SEQ ID NO: 110) or a cysteine residue substituted in place of the native aspartate at position 247 (Gel<sub>C247A44A50</sub>, shown in SEQ ID NO: 111).

The variants of recombinant gelonin were constructed by restriction fragment manipulation or by overlap extension PCR with synthetic oligonucleotides. The sequences of the primers used for PCR are set out below. In each mutagenic primer sequence, the nucleotides corresponding to the changed amino acid, either a cysteine or an alanine residue, are underlined.

Gelo-9 (SEQ ID NO: 20)

Gelo-11 (SEQ ID NO: 18)

Gelo-16 (SEQ ID NO: 25)

Gelo-17 (SEQ ID NO: 27)

Gelo-18 (SEQ ID NO: 26)

Gelo-19 (SEQ ID NO: 58)

5' CAGCCATGGAATCCCATTGCTG 3'

GeloC-1 (SEQ ID NO: 28)

5' TCGATTGCGATCCTAAATAGTACTC 3'

GeloC-2 (SEQ ID NO: 29)

5' TTTAGGATCGCAATCGACGAACTTCAAG 3'

GeloC-3-2 (SEQ ID NO: 30)

5' GTTCGTCTGTAAAGATCCTAAATAGTACTCGA 3'

GeloC-4 (SEQ ID NO: 31)

5' GGATCTTTACAGACGAACTTCAAGAGT 3'

-45-

GeloC-5 (SEQ ID NO: 32)

5' TCTTGTGCTTCGTCGATAAAGATCC 3'

GeloC-6 (SEQ ID NO: 33)

5' ATCGACGAAGCACAAGAGTGCTATTTT 3'

5

GeloC-9 (SEQ ID NO: 34)

5' GTAAACCATGCATAGCACTCTTGAAGTTCGT 3'

GeloC-10 (SEQ ID NO: 35)

5' AGTGCTATGCATGGTTTTACTTGATCAACTGC 3'

10

GeloC-13 (SEQ ID NO: 36)

5' AGCACATGTGGTGCCACTTATATTACCTA 3'

GeloC-14 (SEQ ID NO: 37)

5' TAAGTGGCACCACATGTGCTAAAGCTCACGGTG 3'

GeloC-15 (SEQ ID NO: 38)

5' TGA CTGTGGACAGTTGGCGGAAATA 3'

15

GeloC-16 (SEQ ID NO: 39)

5' GCCAACTGTCCACAGTCATTTGAAAGCGCTACC 3'

GeloC-17 (SEQ ID NO: 40)

5' GATGATCCTGGAAAGGCTTTCGTTTTGGTAGCGCTT3'

GeloC-18 (SEQ ID NO: 41)

20

5' AAGCCTTTCCAGGATCATCAGC

TTTTTGGCGCAGCAATGGG 3'

GeloC-19 (SEQ ID NO: 42)

5' AAGCCTTTCCAGGATCATCACAT 3'

GeloC-20 (SEQ ID NO: 59)

25

5' CACATGTAAACAAGACTTCATTTTGGC 3'

-46-

GeloC-21 (SEQ ID NO: 60)

5' TGAAGTCTTGTTTTAGATGTGTTTTGAAGAGGCCT3'

GeloC-22 (SEQ ID NO: 61)

5' ATGCCATATGCAATTATAAACCAACGGAGA 3'

5

GeloC-23 (SEQ ID NO: 62)

5' GGTTTATAATTGCATATGG

CATTTTCATCAAGTTTCTTG 3'

GeloC-24 (SEQ ID NO: 63)

5' CTTTCAACAATGCAATTCGCCGGCGAATAATAC 3'

10

GeloC-25 (SEQ ID NO: 64)

5' GCGAATGCATTGTTGAAAGTTATTTCTAATTTG 3'

GeloC-26 (SEQ ID NO: 65)

5' GTTTTGTGAGGCAGTTGAATTGGAAC 3'

15

GeloC-27 (SEQ ID NO: 66)

5' TTCAACTGCCTCACAAAACATTCCATTTGCACCT 3'

GeloC-28 (SEQ ID NO: 67)

5' AAAAGCTGATGATCCTGGAAAGTG 3'

GeloC-29 (SEQ ID NO: 68)

5' TCCAGGATCATCAGCTTTTTTGCGCAGCAATGGGA 3'

20

araB2 (SEQ ID NO: 43)

5' GCGACTCTCTACTGTTTC 3'

HINDIII-2 (SEQ ID NO: 44)

5' CGTTAGCAATTTAACTGTGAT 3'

25

(1) Specifically, a cysteine was introduced at amino acid 247 of gelonin (which is normally occupied by an aspartic acid which corresponds to the cysteine at position

-47-

259 in the ricin A-chain) by PCR with mutagenic primers GeloC-3-2 and GeloC-4 in conjunction with primers *HINDIII*-2 (a primer located in the vector portion of pING3734 or pING3825), Gelo-9 and Gelo-8. Template DNA (pING3734) was amplified with GeloC-3-2 and *HINDIII*-2 and in a concurrent reaction with GeloC-4 and Gelo-9. The products of these reactions were mixed and amplified with the outside primers Gelo-8 and *HINDIII*-2. The reaction product was cut with *EcoRI* and *XhoI*, purified, and was inserted into plasmid pING3825 in a three-piece ligation. The DNA sequence of the Gel<sub>C247</sub> variant (SEQ ID NO: 102) was then verified. The plasmid containing the sequence encoding Gel<sub>C247</sub> was designated pING3737 and was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 on June 9, 1992 as ATCC Accession No. 69009.

(2-3) In the same manner, a cysteine residue was introduced in place of the amino acid at position 248 (a lysine) of gelonin with the mutagenic oligonucleotides GeloC-1 and GeloC-2 to generate analog Gel<sub>C248</sub> (SEQ ID NO: 103) in plasmid pING3741, and a cysteine residue was introduced at amino acid position 239 (normally occupied by a lysine) with primers GeloC-9 and GeloC-10 to generate analog Gel<sub>239</sub> (SEQ ID NO: 104) in plasmid pING3744.

(4) Also in the same manner, a cysteine residue was introduced at amino acid 244 (a lysine) of gelonin with mutagenic primers GeloC-5 and GeloC-6 to generate analog Gel<sub>C244</sub> (SEQ ID NO: 105) in a plasmid designated pING3736. This variant was prepared by PCR using plasmid pING3734 as template DNA rather than pING3825. It therefore encodes the same N-terminal gelonin amino acid sequence as plasmids pING3737, pING3741, and pING3744, but includes the PCR primer-derived 5'-end 32 nucleotides instead of the native gelonin 5'-end nucleotides.

(5) A cysteine residue was introduced in place of the amino acid (normally occupied by a lysine) at position 10 of gelonin by a similar procedure. A cysteine was introduced with mutagenic primers GeloC-13 and GeloC-14 by

-48-

amplifying pING3824 with araB2 (a vector primer) and GeloC-14, and in a separate reaction, with GeloC-13 and Gelo-11. ~~These reaction products were mixed and amplified with the~~ outside primers araB2 and Gelo-11. The PCR product was cut with *Pst*I and *Nco*I, purified, and cloned back into pING3825 to generate analog Gel<sub>C10</sub> (SEQ ID NO: 106) in the plasmid designated pING3746 and deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 on June 9, 1992 as ATCC Accession No. 69008.

(6) The asparagine at position 60 of gelonin was replaced with a cysteine residue using two mutagenic oligos, GeloC-15 and GeloC-16, in conjunction with oligos araB2 and Gelo-11 in the same manner as for the Gel<sub>C10</sub> variant. The plasmid encoding the Gel<sub>C60</sub> (SEQ ID NO: 107) analog was designated pING3749.

(7) A cysteine was introduced at amino acid 103 (an isoleucine) by PCR with mutagenic primers GeloC-20 and GeloC-21 in conjunction with primers araB2 and *HIND*III-2. Template DNA (pING3733) was amplified with GeloC-21 and araB2 and separately with GeloC-20 and *HIND*III-2. The products of these reactions were mixed and amplified with the outside primers araB2 and *HIND*III-2. The reaction product was cut with *Nco*I and *Bcl*I, purified, and inserted into pING3825 digested with *Nco*I and *Bcl*I. The oligonucleotides used to place a cysteine at residue 103 also introduced an *Afl*III restriction site which was verified in the cloned gene. The plasmid containing the Gel<sub>C103</sub> (SEQ ID NO: 108) analog was designated pING3760.

(8) A cysteine was introduced at position 146 (an aspartic acid) by a similar strategy. Template DNA (pING3733) was amplified with mutagenic primer GeloC-22 and Gelo-14 and separately with mutagenic primer GeloC-23 and Gelo-19. The products of these reactions were mixed, and amplified with Gelo-19 and Gelo-14. The reaction product was cut with *Bgl*II and *Eco*RI, and can be inserted into pING3825 in a three-piece ligation. The oligonucleotides used to place a cysteine at residue 146 also introduced a

-49-

*Nde*I restriction site which can be verified in the cloned gene.

(9) To introduce a cysteine at position 184 (normally occupied by an arginine) of gelonin, template DNA (pING3733) was amplified with mutagenic primer GeloC-25 and araB-2 and separately with mutagenic primer GeloC-24 and *HIND*III-2. The products of these reactions were mixed, and amplified with araB2 and Gelo-14. The reaction product was cut with *Nco*I and *Bcl*I, and inserted into pING3825 previously digested with *Nco*I and *Bcl*I. The oligonucleotides used to place a cysteine at residue 184 also introduced an *Nsi*I restriction site which was verified in the cloned gene. The plasmid containing the sequence encoding the Gel<sub>C184</sub> (SEQ ID NO: 109) variant was designated pING3761.

(10) A cysteine may be introduced at position 215 (a serine) by a similar strategy. Template DNA (pING3733) was amplified with mutagenic primer GeloC-27 and araB2 and separately with mutagenic primer GeloC-26 and *HIND*III-2. The products of these reactions were mixed, and amplified with araB2 and *HIND*III-2. The reaction product was cut with *Eco*RI and *Bcl*I, and may be inserted into pING3825 in a three-piece ligation.

(11) Another gelonin variant with a free cysteine residue was generated by replacing one of the two naturally occurring gelonin cysteine residues, the cysteine at position 50, with an alanine. Plasmid pING3824 was amplified with primers GeloC-17 and Gelo-11, and concurrently in a separate reaction with primers GeloC-19 and araB2. The reaction products were mixed and amplified with araB2 and Gelo-11. This product was cut with *Nco*I and *Bgl*II, and cloned back into the vector portion of pING3825 to generate pING3747 (ATCC 69101). This analog was designated Gel<sub>A50(C44)</sub> and it contains a cysteine available for disulfide bonding at amino acid position 44. Non-cysteine residues, other than alanine, which do not disrupt the activity of gelonin, also may be inserted at position 50 in

-50-

natural gelonin in order to generate a gelonin analog with a single cysteine at position 44.

(12) A gelonin variant in which the natural cysteine at position 44 was changed to alanine was constructed by amplifying pING3733 using the mutagenic oligonucleotides GeloC-28 and GeloC-29 in conjunction with primers araB2 and HINDIII-2. The amplified DNA was cut with NcoI and BglII and cloned into a gelonin vector, generating pING3756. That variant generated was designated Gel<sub>A44(C50)</sub>. Non-cysteine residues, other than alanine, which do not disrupt gelonin activity, also may be inserted at position 44 in order to generate a gelonin analog with a single cysteine at position 50.

(13) A gelonin variant in which both the cysteine at position 44 and the cysteine at position 50 of gelonin were changed to alanine residues was constructed by overlap PCR of pING3824 using the mutagenic oligonucleotides GeloC-17 and GeloC-18 in conjunction with primers araB2 and Gelo-11. This analog, like the native gelonin protein, has no cysteine residues available for conjugation. The plasmid encoding the analog was designated pING3750. The analog generated was designated Gel<sub>A44A50</sub> (SEQ ID NO: 101). Non-cysteine residues, other than alanine, which do not disrupt gelonin activity, also may be substituted at both positions 44 and 50 in order to generate a gelonin analog with no cysteine residues.

(14) The triple mutant Gelonin<sub>C247A44A50</sub> (SEQ ID NO: 111) was constructed from the plasmids pING3824, pING3750 and pING3737. This variant contains an introduced cysteine at position 247 while both of the naturally occurring cysteine residues at positions 44 and 50 have been replaced with alanine. The analog is desirable because, in this analog, disulfide linkage to an antibody is only assured at a single cysteine residue. Plasmid pING3824 was cut with NcoI and XhoI and the vector fragment was purified in an agarose gel. pING3750 was cut with NcoI and EcoRI and pING3737 was cut with EcoRI and XhoI. The NcoI-EcoRI



-51-

fragment encodes the alanines at positions 44 and 50 while the *EcoRI-XhoI* fragment encodes the cysteine at position 247. Each of these fragments was purified and ligated to the *NcoI* to *XhoI* vector fragment. The resulting plasmid is named pING3752.

(15) The triple mutant Gelonin<sub>C10A44A50</sub> (SEQ ID NO: 110) was also constructed by assembly from previously assembled plasmids. In this case, pING3746 was cut with *PstI* and *NcoI*, while pING3750 was cut with *NcoI* and *XhoI*. Each of the insert fragments were purified by electrophoresis in an agarose gel, and the fragments were ligated into a *PstI* and *XhoI* digested vector fragment. The resulting vector was designated pING3753. The Gel<sub>C10A44A50</sub> analog has been referred to previously as Gel<sub>C10C44AC50A</sub> (see, e.g., co-owned, co-pending U.S. Patent Application Serial No. 07/988,430, incorporated by reference herein).

Each of the gelonin variants constructed was transformed into *E. coli* strain E104. Upon induction of bacterial cultures with arabinose, gelonin polypeptide could be detected in the culture supernatants with gelonin-specific antibodies. There were no significant differences detected in the expression levels of gelonin from plasmids pING3734 and pING3825, or in the levels from any of the gelonin variants. Each protein was produced in *E. coli* at levels of approximately 1 g/l.

#### Example 4

##### Reticulocyte Lysate Assay

The ability of gelonin and recombinant gelonin analogs to inhibit protein synthesis *in vitro* was tested using a reticulocyte lysate assay (RLA) described in Press et al., *Immunol. Letters*, 14:37-41 (1986). The assay measures the inhibition of protein synthesis in a cell-free system using endogenous globin mRNA from a rabbit red blood cell lysate. Decreased incorporation of tritiated leucine (<sup>3</sup>H-Leu) was measured as a function of toxin concentration. Serial log dilutions of standard toxin (the 30 kD form of

-52-

ricin A-chain, abbreviated as RTA 30), native gelonin, recombinant gelonin (rGelonin or rGel) and gelonin analogs were tested over a range of 1  $\mu$ g/ml to 1 pg/ml. Samples were tested in triplicate, prepared on ice, incubated for 30 minutes at 37°C, and then counted on an Inotec Trace 96 cascade ionization counter. By comparison with an uninhibited sample, the picomolar concentration of toxin (pM) which corresponds to 50% inhibition of protein synthesis ( $IC_{50}$ ) was calculated. As is shown in Table 1 below, recombinant gelonin and most of its analogs exhibit activity in the RLA comparable to that of native gelonin. For some of the analogs (such as Gel<sub>C239</sub>), RLA activity was diminished.

Table 1

	<u>Toxin</u>	<u>IC<sub>50</sub>(pM)</u>
15	RTA 30	2.5
	Gelonin	15
	rGelonin	11
	Gel <sub>C10</sub>	60
20	Gel <sub>A50(C44)</sub>	20
	Gel <sub>A44(C50)</sub>	47
	Gel <sub>C60</sub>	26
	Gel <sub>C239</sub>	955
	Gel <sub>C244</sub>	32
25	Gel <sub>C247</sub>	12
	Gel <sub>C248</sub>	47
	Gel <sub>A44A50</sub>	16
	Gel <sub>C10A44A50A</sub>	7
	Gel <sub>C247A44A50</sub>	20

-53-

Example 5

## Human-Engineered Antibodies

For Construction-Of-Immunotoxins

Antibodies for use in constructing immunotoxins according to the present invention may be humanized antibodies, such as he3 and fragments thereof which display increased content of human amino acids and a high affinity for human CD5 cell differentiation marker. he3 is a humanized form of a mouse H65 antibody (H65 is a preferred monoclonal antibody for use in preparing humanized antibodies according to the present invention and is produced by hybridoma cell line XMMLY-H65 (H65) deposited with the American Type Culture Collection in Rockville, Maryland (A.T.C.C.) and given the Accession No. HB9286).

Humanized antibodies for use in the present invention are prepared as disclosed herein using the humanized forms of the murine H65 antibody in which both low and moderate risk changes described below were made in both variable regions. Such humanized antibodies should have less immunogenicity and have therapeutic utility in the treatment of autoimmune diseases in humans. For example, because of their increased affinity over existing therapeutic monoclonal antibodies such as H65, he3 antibodies of the invention may be administered in lower doses than H65 anti-CD5 antibodies in order to obtain the same therapeutic effect.

Humanized antibodies, such as he3, are useful in reducing the immunogenicity of foreign antibodies and also results in increased potency when used as a portion of an immunoconjugate.

Construction of humanized antibody variable domains according to the present invention and for use as components of immunotoxins may be based on a method which includes the steps of: (1) identification of the amino acid residues of an antibody variable domain which may be modified without diminishing the native affinity of the domain for antigen while reducing its immunogenicity with respect to a heterologous species; and (2) the preparation

-54-

of antibody variable domains having modifications at the identified residues which are useful for administration to heterologous species. The methods of the invention are based on a model of the antibody variable domain described herein which predicts the involvement of each amino acid in the structure of the domain.

Unlike other methods for humanization of antibodies, which advocate replacement of the entire classical antibody framework regions with those from a human antibody, the methods described herein introduce human residues into the variable domain of an antibody only in positions which are not critical for antigen-binding activity and which are likely to be exposed to immunogenicity-stimulating factors. The present methods are designed to retain sufficient natural internal structure of the variable domain so that the antigen-binding capacity of the modified domain is not diminished in comparison to the natural domain.

The human consensus sequences in which moderate risk residues are converted from mouse residues to human residues are represented in Figures 10A and 10B as lines labelled hK1 (i.e., subgroup 1 of the human kappa chain) and hH3 (i.e., subgroup 3 of the human heavy chain). Symbols in the figures for conservation and for risk in "bind" and "bury" lines are follows:

First Symbol in Pair (Ligand Binding)

- + Little or not direct influence on antigen-binding loops, low risk if substituted
- Indirectly involved in antigen-binding loop structure, moderate risk if changed
- Directly involved in antigen-binding loop conformation or antigen contact, great risk if modified

-55-

## Second Symbol in Pair (Immunogenicity/Struture)

- 5 + Highly accessible to solvent, high immunogenicity, low risk if substituted
- ° Partially buried, moderate immunogenicity, moderate risk if altered
- Completely buried in subunit's hydrophobic core, low immunogenicity, high risk if changed
- 10 = Completely buried in interface between subunits, low immunogenicity, high risk if modified

## Significance of Pairs

- ++ Low risk  
Highly accessible to solvent  
15 and high immunogenicity, but little or no effect on specific antigen binding
- °+, +°, °° Moderate Risk  
Slight immunogenicity or indirect  
20 involvment with antigen binding
- any - or = High risk  
Buried within the subunit core/  
interface or strongly involved in  
antigen binding, but little immunogenic  
25 potential

In the line labelled "mod", a dot (.) represents a residue which may be mutated from "mouse" to "human" at moderate risk. There are 29 such moderate risk positions.

30 The mouse residue matches the human consensus residue more than 50% of the time at 131 positions (102 positions match 90%-100% and 29 positions match 50% to 90%). These positions were not changed.

35 The lines labelled M/H in Figures 12A and 12B indicate the 91 positions which differed significantly between the mouse and human sequences (i.e., where the

-56-

human sequences have the mouse residue less than 50% of the time). Moderate risk positions, designated m in the M/H line, were kept "mouse"; whereas those designated H or h were changed to human. The 25 low risk positions which  
5 were already human-like or which were previously humanized (as described *supra* in Example 2) are designated " ^ " in the M/H line. Finally, the 54 high risk positions in which the mouse and human residues did not match are designated M and are kept "mouse".

10 Fifteen differences occur at moderate risk positions at which the mouse and human sequences differ. At ten of those positions (designated "H" on the M/H lines of Figure 6) the mouse residue aligns with a human consensus amino acid which is highly conserved. Therefore,  
15 the mouse residue at that position is identified as one to be changed to the conserved human residue.

At moderate risk positions (designated "m") in which the mouse and the human sequences differ, the mouse residue aligns with a human consensus amino acid which is  
20 moderately conserved. However, since the mouse residue is found at that position in other actual sequences of human antibodies [See Kabat, et al., sequences of Proteins of Immunoglobulin Interest, Fourth Edition, U.S. Department of Health and Human Services, Public Health Service, National  
25 Institutes of Health (1987)] the positions are identified as ones to be kept "mouse." Although there are no such positions in this particular sequence, such positions may occur in other antibodies.

At four moderate risk positions (designated "h"),  
30 the mouse residue aligns with a human consensus amino acid which is moderately conserved but the mouse residue is not found at that position in an actual human antibody sequence in Kabat, et al. *Sequences of Proteins of Immunoglobulin Interest, supra*. Therefore, that position is identified as  
35 ones to be changed to "human."

At one moderate risk position (designated "m") in which the mouse and human sequences differ, the mouse

-57-

residue aligns with a human consensus amino acid which is poorly conserved. Therefore, that position is identified as one to be kept "mouse."

A. Assembly Of Moderate Risk

5       Heavy Chain Expression Vectors

10       The humanized H65 heavy chain containing the moderate risk residues was assembled by the following strategy. The moderate-risk expression vector was assembled from intermediate vectors. The six  
15       oligonucleotide sequences (oligos), disclosed in Figure 12 and labelled HUH-G11, HUH-G12, HUH-G3, HUH-G4, HUH-G5, and HUH-G6 (the sequences of HUH-G11 and HUH-G12 are set out in SEQ ID Nos. 131 and 132 and HUH-G3, HUH-G4, HUH-G5, and HUH-G6 are set out in SEQ ID NOS: 137-140) were assembled  
20       by PCR. Oligonucleotides containing the synthetic humanized antibody gene were mixed in pairs (HUH-G11 + HUH-G12, HUH-G3 + HUH-G4, and HUH-G5 + HUH-G6) in a 100  $\mu$ l reaction with 1  $\mu$ g of each DNA and filled in as described above. A portion of each reaction product was mixed in  
25       pairs (HUH-G11, 12 + HUH-G3, 4; HUH-G3, 4 + HUH-G5, 6), 2.5 U Taq was added and samples were reincubated as described above. The V-J region was assembled by mixing equal amounts of the HUH-G11, 12, 3, 4 reaction product with the HUH-G3, 4, 5, 6 product, followed by PCR with 0.5  $\mu$ g of  
30       primers H65G-2S and H65-G2 as described above. The reaction product was cut with *Sal*I and *Bst*EII and cloned into the expression vector, similar to that described for heavy chain in Robinson et al., *Hum. Antibod. Hybridomas* 2:84 (1991), generating pING4617. That plasmid was  
35       sequenced with Sequenase (USB, Cleveland), revealing that two residues were altered (a G-A at position 288 and a A-T at position 312, numbered from the beginning of the leader sequence). The correct variable region was restored by substitution of this region from pING4612, generating the expected V-region sequence in pING4619.

An intermediate vector containing the other moderate-risk changes was constructed by PCR assembly of

-58-

the oligos HUH-G13, HUH-G14, HUH-G15, and HUH-G16 (Fig. 11 and SEQ ID Nos: 133-136). Oligos HUH-G13 + HUH-G14 and ~~HUH-G15 + HUH-G16~~ were mixed and filled in with Vent polymerase (New England Biotabs) in a reaction containing

5 10 mM KCl, 20 mM TRIS pH 8.8, 10 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2mM  $\text{MgSO}_4$ , 0.1% Triton X-100, 100 ng/ml BSA, 200  $\mu\text{M}$  of each dNTP, and 2 units of Vent polymerase in a total volume of 100  $\mu\text{l}$ . The reaction mix was incubated at 94°C for 1 minute, followed by 2 minutes at 50°C and 20 minutes at 72°C. The

10 reaction products (40  $\mu\text{l}$ ) were mixed and amplified with the oligonucleotides H65-G13 and H65-G2 with Vent polymerase in the same reaction buffer and amplified for 25 cycles with denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes and polymerization at 72°C for 3 minutes. The

15 reaction product was treated with T4 polymerase and then digested with AccI. The 274 base pair (bp) fragment was purified on an agarose gel and ligated along with the 141 bp SalI to AccI fragment from pING4619 into pUC18 cut with SalI and SmaI to generate pING4620. pING4620 contains the

20 entire signal sequence, V-region, and J-region of the moderate-risk H65 heavy chain.

The final expression vector for the moderate-risk H65 heavy chain, pING4621, was assembled by cloning the SalI to BstEII fragment from pING4620 into the same

25 expression vector described above.

#### B. Assembly Of Moderate-Risk Light Chain Expression Vectors

The moderate-risk humanized V- and J-segments of the light chain were assembled from six oligonucleotides,

30 \$H65K-1 (SEQ ID NO: 117), HUH-K7 (SEQ ID NO: 119), HUH-K6 (SEQ ID NO: 118), HUH-K8 (SEQ ID NO: 120), HUH-K4 (SEQ ID NO: 121 and HUH-K5 (SEQ ID NO: 122). The oligonucleotides were amplified with PCR primers H65K-2S and JK1-HindIII. Oligonucleotides containing the synthetic humanized

35 antibody gene were mixed in pairs (\$H65-K1 + HUH-K7, HUH-K6 + HUH-K4 + HUH-K5) and incubated with Vent polymerase as described for the moderate-risk heavy chain. A portion of



-59-

each reaction product (40 ul) was mixed in pairs (\$H65H-K1/HUH-K7 + HUH-K6, 8; HUH-K6, 8 + HUH-K4, 5) and filled in as above. The light chain gene was then assembled by amplifying the full length gene with the PCR primers H65K-2S and JK1-HindIII with Vent polymerase for 25 cycles as outlined above. The assembled V/J region was cut with SalI and HindIII, purified by electrophoresis on an agarose gel, and assembled into a light chain antibody expression vector, pING4630.

10

#### Example 6

##### Transfection Of he3 Genes And Purification Of Expression Products

##### A. Stable Transfection Of Mouse Lymphoid Cells For The Production Of he3 Antibody

15

The cell line Sp2/0 (American Type Culture Collection Accession No. CRL1581) was grown in Dulbecco's Modified Eagle Medium plus 4.5 g/l glucose (DMEM, Gibco) plus 10% fetal bovine serum. Media were supplemented with glutamine/penicillin/streptomycin (Irvine Scientific, Irvine, California).

20

The electroporation method of Potter, H., et al., Proc. Natl. Acad. Sci., USA, 81:7161 (1984) was used. After transfection, cells were allowed to recover in complete DMEM for 24-48 hours, and then seeded at 10,000 to 50,000 cells per well in 96-well culture plates in the presence of selective medium. Histidinol (Sigma) selection was at 1.71 µg/ml, and mycophenolic acid (Calbiochem) was at 6 µg/ml plus 0.25 mg/ml xanthine (Sigma). The electroporation technique gave a transfection frequency of 1-10 x 10<sup>-5</sup> for the Sp2/0 cells.

25

30

The he3 light chain expression plasmid pING4630 was linearized by digestion with PvuI restriction endonuclease and transfected into Sp2/0 cells, giving mycophenolic acid - resistant clones which were screened for light chain synthesis.

35

Four of the top-producing subclones, secreting 4.9-7.5 µg/ml were combined into two pools (2 clones/pool)

-60-

and each pool was transfected with plasmid pING42621, containing the moderate-risk heavy chain. After selection with histidinol, the clones producing the most light plus heavy chain, Sp2/0-4630 and 4621 Clones C1705 and C1718, secreted antibody at approximately 15 and 22  $\mu\text{g}/\text{ul}$  respectively in the presence of  $10^{-7}$  M dexamethasone in an overgrown culture in a T25 flask. Clone C1718 was deposited with the American Type Culture Collection, 1230 Parklawn Drive, Rockville, Maryland, 20852 on December 1, 1992 as ATCC HB 11206. The best producer is a subclone of Clone C1718 which is produced by limiting dilution subcloning of Clone C1718.

B. Purification Of he3 Antibody  
Secreted In Tissue Culture

Sp2/0-4630 + 4621 Clone C1705 cells were grown in culture medium HB101 (Hana Biologics)+ 1% Fetal Bovine Serum, supplemented with 10 mM HEPES, 1x Glutamine-Pen-Strep (Irvine Scientific #9316). The spent medium was centrifuged at about 5,000 x g for 20 minutes. The antibody level was measured by ELISA. Approximately 200 ml of cell culture supernatant was loaded onto a 2 ml Protein A-column (Sigma Chemicals), equilibrated with PBS (buffer 0.15 M NaCl, 5 mM sodium phosphate, 1 mM potassium phosphate, buffer pH 7.2). The he3 antibody was eluted with a step pH gradient (pH 5.5, 4.5 and 2.5). A fraction containing he3 antibody (9% yield) but not bovine antibody, was neutralized with 1 M Tris pH 8.5, and then concentrated 10-fold by Centricon 30 (Amicon) diluted 10-fold with PBS, reconcentrated 10-fold by Centricon 30, diluted 10-fold with PBS, and finally reconcentrated 10-fold. The antibody was stored in 0.25 ml aliquots at  $-20^{\circ}$  C.

C. Affinity Measurements Of he3 IgG For CD5

The affinity of he3 IgG for CD5 was determined using Molt-4M cells, which express CD5 on their surface, and  $^{125}\text{I}$ -labeled chimeric H65 IgG in a competitive binding assay. Culture supernatants from Clone C1705 and C1718 and

-61-

purified IgG from C1705 were used as the sources of he3 IgG.

For this assay, 20  $\mu$ g of chimeric H65 IgG (CH65 IgG) was iodinated by exposure to 100  $\mu$ l lactoperoxidase-glucose oxidase immobilized beads (Enzymobeads, BioRad), 100  $\mu$ l of PBS, 1.0 mCi  $I^{125}$  (Amersham, IMS30), 50  $\mu$ l of 55 mM b-D-glucose for 45 minutes at 23°C. The reaction was quenched by the addition of 20  $\mu$ l of 105 mM sodium metabisulfite and 120 mM potassium iodine followed by centrifugation for 1 minute to pellet the beads.  $^{125}I$ -CH65 IgG was purified by gel filtration using 7 mls of sephadex G25, using PBS (137 mM NaCl, 1.47 mM  $KH_2PO_4$ , 8.1 mM  $Na_2HPO_4$ , 2.68 mM KCl at pH 7.2-7.4) plus 0.1% BSA.  $^{125}I$ -CH65 IgG recovery and specific activity were determined by TCA precipitation.

Competitive binding was performed as follows: 100  $\mu$ l of Molt-4M cells were washed two times in ice-cold DHB binding buffer (Dubellico's modified Eagle's medium (Gibco, 320-1965PJ), 1.0% BSA and 10 mM Hepes at pH 7.2-7.4). Cells were resuspended in the same buffer, plated into 96 v-bottomed wells (Costar) at  $3 \times 10^5$  cells per well and pelleted at 4°C by centrifugation for 5 min at 1,000 rpm using a Beckman JS 4.2 rotor; 50  $\mu$ l of 2X-concentrated 0.1 nM  $^{125}I$ -CH65 IgG in DHB was then added to each well and competed with 50  $\mu$ l of 2X - concentrated CH65 IgG or humanized antibody in DHB at final antibody concentrations from 100 nM to 0.0017 nM. Humanized antibody was obtained from culture supernatants of Sp2/0 clone C1718 which expresses he3 IgG. The concentration of the antibody in the supernatants was established by ELISA using a chimeric antibody as a standard. The concentration of the antibody in the purified preparation was determined by binding was allowed to proceed at 4°C for 5 hrs and was terminated by washing cells three times with 200  $\mu$ l of DHB binding buffer by centrifugation for 5 min at 1,000 rpm. All buffers and operations were at 4°C. Radioactivity was determined by solubilizing cells in 100  $\mu$ l of 1.0 M NaOH and counting in

-62-

a Cobra II auto gamma counter (Packard). Data from binding experiments were analyzed by the weighted nonlinear least squares curve fitting program, MacLigand, a Macintosh version of the computer program "Ligand" from Munson, *Analyt. Biochem.*, 107:220 (1980). Objective statistical criteria (F, test, extra sum squares principle) were used to evaluate goodness of fit and for discriminating between models. Nonspecific binding was treated as a parameter subject to error and was fitted simultaneously with other parameters.

Figure 11, provides data showing relative binding of he3 and CH65 to CD5 on molt-4M cells in a competition binding assay. These results demonstrate that the moderate-risk changes made in he3 IgG result in an antibody with a higher affinity than the chimeric mouse-human form of this antibody (CH65) for its target, CD5.

#### Example 7

##### Preparation of Gelonin Immunoconjugates

Gelonin analogs of the invention were variously conjugated to murine (ATCC HB9286) and chimeric H65 (CH65) antibody, CH65 antibody domains (including cFab, cFab' and cF(ab')<sub>2</sub> fragments), and humanized antibodies and antibody domains, all of which are specifically reactive with the human T cell determinant CD5. H65 antibody was prepared and purified by methods described in U.S. Patent Application Serial No. 07/306,433, *supra* and International Publication No. WO 89/06968, *supra*. Chimeric H65 antibody was prepared according to methods similar to those described in Robinson et al., *Human Antibodies and Hybridomas*, 2:84-93 (1991), incorporated by reference herein. Chimeric H65 Fab, Fab', and F(ab')<sub>2</sub> proteins were prepared as described in Better, et al., *Proc. Nat. Acad. Sci. (USA)*, 90: 457-461 (1993), incorporated by reference herein. Finally, he3 humanized antibodies were prepared according to the procedures described in U.S. Patent

-63-

Application Serial No. 07/808,464, incorporated by reference herein.

A. Conjugation To H65 Antibodies

5 To expose a reactive sulfhydryl, the unpaired cysteine residues of the gelonin analogs were first reduced by incubation with 0.1 to 2 mM DTT (30-60 minutes at room temperature), and then were desalted by size-exclusion chromatography.

Specifically, the Gel<sub>C248</sub> analog (3.8 mg/ml) was  
10 treated with 2 mM DTT for 60 minutes in 0.1 M Naphosphate, 0.25 M NaCl, pH 7.5 buffer. The Gel<sub>C244</sub> variant (7.6 mg/ml) was treated with 2 mM DTT for 30 minutes in 0.1 M Naphosphate, 0.25 M NaCl, pH 7.5 buffer. The Gel<sub>C247</sub> analog (4 mg/ml) was treated with 2 mM DTT for 30 minutes in 0.1  
15 M Naphosphate, 0.5 M NaCl, pH 7.5 buffer with 0.5 mM EDTA. The Gel<sub>C239</sub> variant (3.2 mg/ml) was treated with 2 mM DTT for 30 minutes in 0.1 M Naphosphate, 0.5 M NaCl, pH 7.5 buffer with 0.5 mM EDTA. The Gel<sub>AS0(C44)</sub> analog (4.2 mg/ml) was treated with 0.1 mM DTT for 30 minutes in 0.1 M  
20 Naphosphate, 0.1 M NaCl, pH 7.5 buffer with 0.5 mM EDTA. Lastly, the Gel<sub>C10</sub> variant (3.1 mg/ml) was treated with 1 mM DTT for 20 minutes in 0.1 M Naphosphate, 0.1 M NaCl, pH 7.5 buffer with 1 mM EDTA.

25 The presence of a free sulfhydryl was verified by reaction with DTNB and the average value obtained was  $1.4 \pm 0.65$  SH/molecule. No free thiols were detected in the absence of reduction.

H65 antibody and chimeric H65 antibody were chemically modified with the hindered linker 5-methyl-2-iminothiolane (M2IT) at lysine residues to introduce a  
30 reactive sulfhydryl group as described in Goff et al., *Bioconjugate Chem.*, 1:381-386 (1990) and co-owned Carroll et al., U.S. Patent No. 5,093,475, incorporated by reference herein.

35 Specifically, for conjugation with Gel<sub>C248</sub> and Gel<sub>C244</sub>, murine H65 antibody at 4 mg/mL was derivitized with

-64-

18x M2IT and 2.5 mM DTNB in 25 mM TEOA, 150 mM NaCl, pH 8 buffer for 1 hour at 23°C. The reaction gave 1.9 linkers per antibody as determined by DTNB assay.

5 For conjugation with Gel<sub>C24</sub>, and Gel<sub>C239</sub>, H65 antibody at 4.7 mg/mL was derivitized with 20x M2IT and 2.5 mM DTNB in 25 mM TEOA 150 mM NaCl, pH 8 buffer for 50 minutes at 23°C. The reaction gave 1.6 linkers per antibody as determined by DTNB assay.

10 Before reaction with Gel<sub>A50(C44)</sub>, H65 antibody at 5.8 mg/mL was derivitized with 20x m2IT and 2.5 mM DTNB in 25 mM TEOA, 150 mM NaCl, pH 8 buffer for 30 minutes at 23°C. The reaction gave 1.5 linkers per antibody as determined by DTNB assay.

15 For conjugation with Gel<sub>C10</sub>, H65 antibody at 2.2 mg/mL was derivitized with 10x m2IT and 2.5 mM DTNB in 25 mM TEOA, 150 mM NaCl, pH 8 buffer for 1 hour at 23°C. The reaction gave 1.4 linkers per antibody as determined by DTNB assay.

20 Chimeric H65 antibody was prepared for conjugation in a similar manner to murine H65 antibody.

Two methods were initially compared for their effectiveness in preparing immunoconjugates with recombinant gelonin. First, the native disulfide bond in recombinant gelonin was reduced by the addition of 2mM DTT at room temperature for 30 minutes. The reduced gelonin was recovered by size-exclusion chromatography on a column of Sephadex GF-05LS and assayed for the presence of free  
25 sulfhydryls by the DTNB assay. 1.4 free SH groups were detected. This reduced gelonin was then reacted with H65-  
30 (M2IT)-S-S-TNB (1.8 TNB groups/H65). Under these experimental conditions, little or no conjugate was prepared between reduced gelonin and thiol-activated H65 antibody.

35 In contrast, when both the recombinant gelonin and the H65 antibody were first derivitized with the crosslinker M2IT (creating gelonin-(M2IT)-SH and H65-(M2IT)-S-S-TNB) and then mixed together, H65-(M2IT)-S-S-

-65-

(M2IT)-gelonin conjugate was prepared in good yield (toxin/antibody ratio of 1.6). The starting materials for this conjugation (gelonin-(M2IT)-SH and H65-(M2IT)-S-S-TNB) contained linker/protein ratios of 1.2 and 1.4, respectively. Native gelonin was derivatized in a similar manner prior to conjugation to murine or chimeric H65 antibody.

The reduced gelonin analogs were mixed with H65-(M2IT)-S-S-TNB to allow conjugation. The following conjugation reactions were set up for each analog: 23 mg (in 7.2 ml) of H65-M2IT-TNB were mixed with a 5-fold molar excess of Gel<sub>C248</sub> (23 mg in 6 ml) for 2 hours at room temperature, then for 18 hours overnight at 4°C; 23 mg (in 7.3 ml) of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel<sub>C244</sub> (23 mg in 3 ml) for 3 hours at room temperature, then for 18 hours overnight at 4°C; 9 mg (in 2.8 mL) of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel<sub>C247</sub> (9 mg in 2.25 mL) for 2 hours at room temperature, then for 5 nights at 4°C; 9 mg (in 2.8 mL) of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel<sub>C239</sub> (9mg in 2.6 mL) for 2 hours at room temperature, then at 4°C for 3 days; 12 mg (in 1.9 mL) of H65-m2IT-TNB were mixed with a 5.6-fold molar excess of Gel<sub>A50(C44)</sub> (13.44 mg in 3.2 mL) for 4.5 hours at room temperature, then 4°C overnight; and 11 mg of H65-m2IT-TNB were mixed with a 5-fold molar excess of Gel<sub>C10</sub> (11 mg in 3.5 mL) for 4 hours at room temperature, then at 4°C overnight.

Following conjugation, unreacted M2IT linkers on the antibody were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was then loaded onto a gel filtration column [Sephadex G-150 (Pharmacia) in the case of Gel<sub>C248</sub>, Gel<sub>C247</sub>, Gel<sub>C244</sub> and Gel<sub>C239</sub> and an Aca-44 column (IBF Biotechnics, France) in the case of Gel<sub>A50(C44)</sub> and Gel<sub>C10</sub>]. The reactions were run over the gel filtration columns and eluted with 10 mM Tris, 0.15M NaCl pH 7. The first peak off each column was loaded onto Blue Toyopearl® resin

-66-

(TosoHaas, Philadelphia, Pennsylvania) in 10 mM Tris, 30 mM NaCl, pH 7 and the product was eluted with 10 mM Tris, 0.5 M NaCl, pH 7.5.

Samples of the final conjugation products were run on 5% non-reduced SDS PAGE, Coomassie stained and scanned with a Shimadzu laser densitometer to quantitate the number of toxins per antibody (T/A ratio). The yield of final product for each analog conjugate was as follows: Gel<sub>C248</sub>, 17 mg with a T/A ration of 1.6; Gel<sub>C247</sub>, 1.1 mg with a T/A ratio of 1; Gel<sub>C244</sub>, 4.5 mgs with a T/A ratio of 1.46; Gel<sub>C239</sub>, 2.9 mg with a T/A ratio of 2.4; Gel<sub>A50(C44)</sub>, 7.3 mg with a T/A ratio of 1.22; and Gel<sub>C10</sub>, 6.2 mg with a T/A ratio of 1.37. Conjugation efficiency (i.e., conversion of free antibody to immunoconjugate) was significantly greater (~80%) for some analogs (Gel<sub>C10</sub>, Gel<sub>A50(C44)</sub>, Gel<sub>C239</sub>, Gel<sub>C247</sub>, and Gel<sub>C248</sub>) than for others (~10%, Gel<sub>C244</sub>).

#### B. Gelonin Immunoconjugates With Chimeric And Humanized Antibodies

Analogues Gel<sub>C247</sub> and Gel<sub>A50(C44)</sub> were also conjugated to various chimeric [CH65Fab, CH65Fab' and CH65F(ab')<sub>2</sub>] and "human engineered" [he1 Fab, he2-Fab, he3-Fab, he1 Fab' and he1 F(ab')<sub>2</sub>] antibody fragments. Chimeric H65 antibody fragments may be prepared according to the methods described in International Publication No. WO 89/00999, supra. The DNA sequences encoding the variable regions of H65 antibody fragments that were human engineered (referring to the replacement of selected murine-encoded amino acids to make the H65 antibody sequences less immunogenic to humans) according to the methods described above in Example 5, are set out in SEQ ID NO: 69 (the kappa chain of he1 and he2), SEQ ID NO: 70 (the gamma chain of he1), SEQ ID NO: 71 (the gamma chain of he2 and he3) and SEQ ID NO: 72 (the kappa chain of he3).

The chimeric H65 antibody fragments were conjugated to the Gel<sub>C247</sub> analog in the same manner as



-67-

described below for conjugation of human engineered Fab and Fab' fragments to Gel<sub>C247</sub> and Gel<sub>A50(C44)</sub>.

(i) hel Fab-Gel<sub>C247</sub>

5 The hel Fab was dialyzed into 25 mM TEOA buffer, 250 mM NaCl, pH 8 and then concentrated to 6.8 mg/mL prior to derivitization with the M2IT crosslinker. For the linker reaction, M2IT was used at 20-fold molar excess, in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 30 minutes at room temperature, then desalted on GF05 (gel filtration resin) and equilibrated in 0.1 M Na phosphate, 0.2M NaCl, pH 7.5. A linker number of 1.8 linkers per Fab was calculated based on the DTNB assay. The hel Fab-M2IT-TNB was concentrated to 3.7 mg/mL prior to conjugation with Gel<sub>C247</sub>.

15 Gel<sub>C247</sub> at 12.8 mg/mL in 10 mM Na phosphate, 0.3M NaCl, was treated with 1 mM DTT, 0.5 mM EDTA for 20 minutes at room temperature to expose a reactive sulfhydryl for conjugation and then was desalted on GF05 and equilibrated in 0.1 M Na phosphate, 0.2 M NaCl, pH 7.5. Free thiol content was determined to be 0.74 moles of free SH per mole of Gel<sub>C247</sub> using the DTNB assay. The gelonin was concentrated to 8.3 mg/mL prior to conjugation with activated antibody.

25 The conjugation reaction between the free thiol on Gel<sub>C247</sub> and the derivitized hel Fab-M2IT-TNB, conditions were as follows. A 5-fold excess of the gelonin analog was added to activated hel Fab-M2IT-TNB (both proteins were in 0.1M Na phosphate, 0.2M NaCl, pH7.5) and the reaction mixture was incubated for 3.5 hours at room temperature and then overnight at 4°C. Following conjugation, untreated M2IT linkers were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was loaded onto a gel filtration column (G-75) equilibrated with 10 mM Tris, 150 mM NaCl, pH 7. 35 The first peak off this column was diluted to 30 mM NaCl

-68-

with 10 mM Tris, pH7 and loaded on Blue Toyopearl®. The product was eluted with 10 mM Tris, 0.5 M NaCl, pH 7.5.

(ii) hel Fab'-Gel<sub>C247</sub>

Similarly, the H65 hel Fab' fragment was dialyzed into 25 mM TEOA buffer, 400 mM NaCl, pH 8 at 2.9 mg/mL prior to derivitization with the M2IT crosslinker. For the linker reaction, M2IT was used at 20-fold molar excess, in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 1 hour at room temperature then it was desalted on GF05 (gel filtration resin) and equilibrated in 0.1 M Na phosphate, 0.2 M NaCl, pH 7.5. A linker number of 1.6 linkers per Fab' was calculated based on the DTNB assay. The hel Fab'-M2IT-TNB was concentrated to 3.7 mg/mL prior to conjugation with Gel<sub>C247</sub>

The Gel<sub>C247</sub> at 77 mg/mL was diluted with 10 mM Na phosphate, 0.1 M NaCl to a concentration of 5 mg/mL, treated with 1 mM DTT, 0.5 mM EDTA for 30 minutes at room temperature to expose a free thiol for conjugation and then was desalted on GF05 and equilibrated in 0.1 M Na phosphate, 0.2 M NaCl, pH 7.5. Free thiol content was determined to be 1.48 moles of free SH per mole of Gel<sub>C247</sub> using the DTNB assay. The Gel<sub>C247</sub> was concentrated to 10 mg/mL prior to conjugation with activated hel Fab'-M2IT-TNB.

For the reaction between the free thiol on Gel<sub>C247</sub> and the derivitized hel Fab'-M2IT-TNB, conditions were as follows. A 5.7-fold molar excess of gelonin was added to activated hel Fab'-M2IT-TNB and the final salt concentration was adjusted to 0.25 M. The reaction mix was incubated for 1.5 hours at room temperature and then over the weekend at 4°C. Following conjugation, unreacted M2IT linkers were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was loaded onto a gel filtration column (ACA54) equilibrated with 10 mM Tris, 250 mM NaCl, pH 7.5. The first peak off this column was diluted to 20 mM NaCl with

-69-

10 mM Tris, pH 7 and loaded on Blue Toyopearl® which was equilibrated in 10 mM Tris, 20 mM NaCl, pH 7. The column was then washed with 10 mM Tris, 30 mM NaCl, pH 7.5. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5.

5                    (iii) he2-Fab Gel<sub>A50(C44)</sub>

The he2-Fab was dialyzed overnight into 25 mM TEOA, 0.25 M NaCl, pH 8 buffer and then concentrated to 13.3 mg/mL prior to derivitization with the M2IT crosslinker. For the linker reaction, M2IT was used in a  
10                    20-fold molar excess in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 20 minutes at room temperature and was then desalted on a GF05-LS (gel filtration) column, equilibrated in 0.1 M Na phosphate, 0.2 M NaCl with 0.02% Na azide. A linker number of 1.7 linkers  
15                    per Fab-M2IT-TNB was calculated based on the DTNB assay. After derivitization and gel filtration, the he2-Fab concentration was 5.2 mg/mL.

Gel<sub>A50(C44)</sub> at 8.33 mg/mL in 10 mM Na phosphate, pH 7.2 was treated with 5 mM DTT and 0.5 mM EDTA for 30  
20                    minutes at room temperature to expose a reactive thiol for conjugation and then was desalted on GF05-LS resin equilibrated in 0.1 M Na phosphate, 0.1 M NaCl with 0.5 mM EDTA plus 0.02% Na azide, pH 7.5. Free thiol content was determined to be 0.83 moles of free SH per mole of Gel<sub>A50(C44)</sub>  
25                    using the DTNB assay. The gelonin was concentrated to 11.4 mg/mL prior to conjugation with activated he2-Fab.

The conjugation reaction conditions between the free thiol on Gel<sub>A50(C44)</sub> and the derivitized he2-Fab-M2IT-TNB were as follows. A 3-fold excess of the gelonin analog was  
30                    added to activated he2-Fab-M2IT-TNB (both proteins were in 0.1 M Na phosphate, 0.1 M NaCl, pH 7.5 but the gelonin solution contained 0.5 mM EDTA as well). The reaction mixture was concentrated to half its original volume, then the mixture was incubated for 4 hours at room temperature  
35                    followed by 72 hours at 4°C. Following the incubation

-70-

period the efficiency of conjugation was estimated at 70-75% by examination of SDS PAGE.

Following conjugation the excess M2IT linkers were quenched by incubation with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction as loaded onto a gel filtration column (G-75) equilibrated in 10 mM Tris, 0.15 M NaCl, pH 7. The first peak off this column was diluted to 30 mM NaCl with 10 mM Tris, pH 7 and loaded onto a Blue Toyopearl® (TosoHaas) column. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5.

(iv) he3-Fab Gel<sub>A50(C44)</sub>

Similarly, the he3-Fab was dialyzed overnight into 25 mM TEOA, 0.25 M NaCl, pH 8 buffer and then concentrated to 5 mg/mL prior to derivitization with the M2IT crosslinker. For the linker reaction, M2IT was used in a 10-fold molar excess in the presence of 2.5 mM DTNB. The reaction was allowed to proceed for 45 minutes at room temperature and was then desalted on a GF05-LS (gel filtration) column, equilibrated in 0.1 M Na phosphate, 0.2 M NaCl with 0.02% Na azide. A linker number of 1 M2IT per Fab-M2IT-TNB was calculated based on the DTNB assay. After derivitization and gel filtration, the he3-Fab concentration was 5.3 mg/mL.

Gel<sub>A50(C44)</sub> at 7.8 mg/mL in 0.1 M Na phosphate, 0.1 M NaCl, pH 7.5 was treated with 1.5 mM DTT and 1 mM EDTA for 30 minutes at room temperature to expose a reactive thiol for conjugation and then was desalted on GF05-LS resin equilibrated in 0.1 M Na phosphate, 0.1 M NaCl plus 0.02% Na azide, pH 7.5. Free thiol content was determined to be 0.66 moles of free SH per mole of Gel<sub>A50(C44)</sub>, using the DTNB assay. The gelonin was concentrated to 5.2 mg/mL prior to conjugation with activated he3-Fab.

The conjugation reaction conditions between the free thiol on Gel<sub>A50(C44)</sub> and the derivitized he3-Fab-M2IT-TNB were as follows. A 5-fold excess of the gelonin analog was

-71-

added to activated he3-Fab-M2IT-TNB (both proteins were in 0.1 M Na phosphate 0.1 M NaCl, pH 7.5). The reaction mixture was incubated for 2 hours at room temperature followed by 72 hours at 4°C. Following the incubated period the efficiency of conjugation was estimated at 70-75% by examination of SDS PAGE.

Following conjugation, the excess M2IT linkers were quenched by incubation with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction was loaded onto a GammaBind G (immobilized protein G affinity resin, obtained from Genex, Gaithersburg, Maryland) equilibrated in 10 mM Na phosphate, 0.15 M NaCl, pH 7. It was eluted with 0.5 M NaOAc, pH 3 and neutralized with Tris. It was dialyzed into 10 mM Tris, 0.15 M NaCl, pH 7 overnight, then diluted to 30 mM NaCl with 10 mM Tris, pH 7 and loaded onto a blue Toyopearl® (TosoHaas) column. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5

#### Example 8

##### Whole Cell Kill Assays

Immunoconjugates prepared with gelonin and gelonin analogs were tested for cytotoxicity against an acute lymphoblastoid leukemia T cell line (HSB2 cells) and against human peripheral blood mononuclear cells (PBMCs). Immunoconjugates of ricin A-chain with H65 antibody (H65-RTA) and antibody fragments were also tested. The ricin A-chain (RTA) as well as the H65-RTA immunoconjugates were prepared and purified according to methods described in U.S. Patent Application Serial No. 07/306,433, *supra* and in International Publication No. WO 89/06968, *supra*.

Briefly, HSB2 cells were incubated with immunotoxin and the inhibition of protein synthesis in the presence of immunotoxin was measured relative to untreated control cells. The standard immunoconjugates H65-RTA (H65 derivitized with SPDP linked to RTA), H65-Gelonin and H65-rGelonin, H65 fragment immunoconjugate, and gelonin immunoconjugate samples were diluted with RPMI without

-72-

leucine at half-log concentrations ranging from 2000 to 0.632 ng/ml. All dilutions were added in triplicate to wells of microtiter plates containing  $1 \times 10^5$  HSB2 cells per well. HSB2 plates were incubated for 20 hours at 37°C and then pulsed with  $^3\text{H}$ -Leu for 4 hours before harvesting. Samples were counted on the Inotec Trace 96 cascade ionization counter. By comparison with an untreated sample, the picomolar concentration (pM) of immunotoxin which resulted in a 50% inhibition of protein synthesis ( $\text{IC}_{50}$ ) was calculated. In order to normalize for conjugates containing differing amounts of toxin or toxin analog, the cytotoxicity data were converted to picomolar toxin (pM T) by multiplying the conjugate  $\text{IC}_{50}$  (in pM) by the toxin/antibody ratio which is unique to each conjugate preparation.

The PMBC assays were performed as described by Fishwild et al., *Clin. and Exp. Immunol.*, 86:506-513 (1991) and involved the incubation of immunoconjugates with PBMCs for a total of 90 hours. During the final 16 hours of incubation,  $^3\text{H}$ -thymidine was added; upon completion, immunoconjugate-induced inhibition of DNA synthesis was quantified. The activities of the H65 and chimeric H65 antibody conjugates against HSB2 cells and PBMC cells are listed in Table 2 below.

-73-

Table 2IC<sub>50</sub> (pM T)

	<u>Conjugate</u>	<u>HSB2 Cells</u>	<u>PBMCs</u>
	H65-RTA	143	459
5	H65-(M2IT)-S-S-(M2IT)-Gelolin	1770	81
	H65-(M2IT)-S-S-(M2IT)-rGelolin	276	75
	H65-(M2IT)-S-S-Gel <sub>C10</sub>	140	28
	H65-(M2IT)-S-S-Gel <sub>A50(C44)</sub>	99	51
	H65-(M2IT)-S-S-Gel <sub>C239</sub>	2328	180
10	H65-(M2IT)-S-S-Gel <sub>C244</sub>	>5000	>2700
	H65-(M2IT)-S-S-Gel <sub>C247</sub>	41	35
	H65-(M2IT)-S-S-Gel <sub>C248</sub>	440	203
	cH65-RTA <sub>30</sub>	60	400
	cH65-(M2IT)-S-S-(M2IT)-Gelolin	1770	140
15	cH65-(M2IT)-S-S-(M2IT)-rGelolin	153	120
	cH65-(M2IT)-S-S-Gel <sub>C239</sub>	>7000	290
	cH65-(M2IT)-S-S-Gel <sub>C247</sub>	34	60
	cH65-(M2IT)-S-S-Gel <sub>C248</sub>	238	860
	H65-(M2IT)-S-S-Gel <sub>AA4(C50)</sub>	338	ND*
20	H65-(M2IT)-S-S-Gel <sub>C247AA4A50</sub>	71	ND*

\* -- Not determined.

Against HSB2 cells, many of the gelonin analog immunoconjugates were significantly more potent than conjugates prepared with native gelonin or recombinant, unmodified gelonin, both in terms of a low IC<sub>50</sub> value, but also in terms of a greater extent of cell kill. Against human PBMCs, the gelonin analog conjugates were at least as active as native and recombinant gelonin conjugates. Importantly, however, some of the conjugates (for example, Gel<sub>C10</sub>, Gel<sub>A50(C44)</sub> and Gel<sub>C247</sub>) exhibited an enhanced potency against PBMCs compared to native and recombinant gelonin conjugates, and also exhibited an enhanced level of cell kill.

The activities of the H65 antibody fragment conjugates against HSB2 cells and PBMC cells are listed in Tables 3 and 4 below, wherein extent of kill in Table 3

-74-

refers to the percentage of protein synthesis inhibited in HSB2 cells at the highest immunotoxin concentration tested (1  $\mu\text{g/ml}$ ).

Table 3

5	IC <sub>50</sub> (pM T)		
	<u>Conjugate</u>	<u>HSB2 Cells</u>	<u>PBMCs</u>
	CH65Fab'-RTA 30	530	1800
	CH65Fab'-rGelolin	135	160
	CH65Fab'-Gel <sub>C247</sub>	48	64
10	CH65F(ab') <sub>2</sub> -RTA 30	33	57
	CH65F(ab') <sub>2</sub> -rGelolin	55	34
	CH65F(ab') <sub>2</sub> -Gel <sub>C247</sub>	23	20
	CH65F(ab') <sub>2</sub> -Gel <sub>C248</sub>	181	95

Table 4

15	IC <sub>50</sub> (pM T)		
	<u>Conjugate</u>	<u>HSB2 Cells</u>	<u>Extent of Kill</u>
	he1 Fab'-Gel <sub>C247</sub>	57.7	93%
	he1 Fab-Gel <sub>C247</sub>	180.0	94%
	he2-Fab-Gel <sub>A50(C44)</sub>	363.0	91%
20	he3-Fab-Gel <sub>A50(C44)</sub>	191.0	93%
	CH65Fab'-Gel <sub>C247</sub>	47.5	93%
	CH65F(ab') <sub>2</sub> -rGelolin	45.4	85%
	CH65F(ab') <sub>2</sub> -Gel <sub>C247</sub>	77.5	83%
	CH65F(ab') <sub>2</sub> -Gel <sub>C247</sub>	23.2	85%

25 The data in Table 3 show that monovalent (Fab or Fab') fragments conjugated to various forms of gelonin are more potent than RTA conjugates. Table 4 shows that the human-engineered gelonin-Fab conjugates exhibit a very high degree of extent of kill.



-75-

Example 9Properties Of Gelonin ImmunoconjugatesA. Solubility

5           Recombinant gelonin and the gelonin analogs  
exhibited enhanced solubility in comparison to both native  
gelonin and RTA30. In addition, recombinant gelonin and  
gelonin analog immunoconjugates exhibited enhanced  
solubility relative to immunoconjugates prepared with  
10       native gelonin and RTA30. This enhanced solubility was  
particularly noteworthy for recombinant gelonin and analog  
conjugates prepared with chimeric Fab fragments.

B. Disulfide Bond Stability Assay

          The stability of the disulfide bond linking a RIP  
to a targeting molecule (such as an antibody) is known to  
15       influence the lifespan of immunoconjugates *in vivo* [See  
Thorpe et al., *Cancer Res.*, 47:5924-5931 (1987),  
incorporated by reference herein]. For example, conjugates  
in which the disulfide bond is easily broken by reduction  
*in vitro* are less stable and less efficacious in animal  
20       models [See Thorpe et al., *Cancer Res.*, 48:6396-6403  
(1988), incorporated by reference herein].

          Immunoconjugates prepared with native gelonin,  
recombinant gelonin and gelonin analogs were therefore  
examined in an *in vitro* disulfide bond stability assay  
25       similar to that described in Wawrzynczak et al., *Cancer  
Res.*, 50:7519-7526 (1990), incorporated by reference  
herein. Conjugates were incubated with increasing  
concentrations of glutathione for 1 hour at 37°C and, after  
terminating the reaction with iodoacetamide, the amount of  
30       RIP released was quantitated by size-exclusion HPLC on a  
TosoHaas TSK-G2000SW column.

          By comparison with the amount of RIP released by  
high concentrations of 2-mercaptoethanol (to determine 100%  
release), the concentration of glutathione required to  
35       release 50% of the RIP (the  $RC_{50}$ ) was calculated. The

-76-

results of assays for H65 antibody conjugates are set out in Table 5 below.

Table 5

	<u>Conjugate</u>	<u>RC<sub>50</sub> (mM)</u>
5	H65-RTA 30	3.2
	H65-(M2IT)-S-S-(M2IT)-gelonin	11.1
	H65-(M2IT)-S-S-(M2IT)-rGelonin	3.0
	H65-(M2IT)-S-S-Gel <sub>C10</sub>	2.5
	H65-(M2IT)-S-S-Gel <sub>A50(C44)</sub>	0.6
10	H65-(M2IT)-S-S-Gel <sub>C239</sub>	774.0
	H65-(M2IT)-S-S-Gel <sub>C244</sub>	1.2
	H65-(M2IT)-S-S-Gel <sub>C247</sub>	0.1
	H65-(M2IT)-S-S-Gel <sub>C248</sub>	0.4
	CH65-RTA 30	2.50
15	CH65-(M2IT)-S-S-(M2IT)-rGelonin	2.39
	CH65-(M2IT)-S-S-Gel <sub>C247</sub>	0.11
	CH65-(M2IT)-S-S-Gel <sub>C248</sub>	0.32
	H65-(M2IT)-S-S-Gel <sub>A44(C50)</sub>	9.2
	H65-(M2IT)-S-S-Gel <sub>C247A44A50</sub>	0.3

20 The foregoing results indicate that the stability of the bonds between the different gelonin proteins and H65 antibody varied greatly. With the exception of Gel<sub>C10</sub> and Gel<sub>C239</sub>, most of the gelonin analogs resulted in conjugates with linkages that were somewhat less stable in the in vitro assay than the dual-linker chemical conjugate. The stability of the Gel<sub>C239</sub> analog, however, was particularly enhanced.

25 The results of the assay for H65 antibody fragment conjugates are set out in Table 6 below.

-77-

Table 6

	<u>Conjugate</u>	<u>RC<sub>50</sub> (mM)</u>
	he1 Fab'-Gel <sub>C247</sub>	0.07
	cFab'-Gel <sub>onin</sub>	1.27
5	cFab'-Gel <sub>C247</sub>	0.08
	cF(ab') <sub>2</sub> -RTA 30	1.74
	cF(ab') <sub>2</sub> -rGel <sub>onin</sub>	2.30
	cF(ab') <sub>2</sub> -Gel <sub>C247</sub>	0.09
	cF(ab') <sub>2</sub> -Gel <sub>C248</sub>	0.32
10	he2-Fab-Gel <sub>A50(C44)</sub>	0.46
	he3-Fab-Gel <sub>A50(C44)</sub>	0.58

From the RC<sub>50</sub> results presented in Tables 5 and 6, it appears that the particular RIP analog component of each immunotoxin dictates the stability of the immunotoxin disulfide bond *in vitro*.

Example 10Pharmacokinetics Of Conjugates To H65 Antibody

The pharmacokinetics of gelonin analogs Gel<sub>C247</sub>, Gel<sub>A50(C44)</sub>, and Gel<sub>C10</sub> linked to whole H65 antibody was investigated in rats. An IV bolus of 0.1 mg/kg of <sup>125</sup>I-labelled immunoconjugate H65-(M2IT)-S-S-Gel<sub>C247</sub>, H65-(M2IT)-S-S-Gel<sub>A50(C44)</sub> or H65-(M2IT)-S-S-Gel<sub>C10</sub> was administered to male Sprague-Dawley rats weighing 134-148 grams. Serum samples were collected from the rats at 3, 15, 30 and 45 minutes, and at 1.5, 2, 4, 6, 8, 18, 24, 48, 72, and 96 hours. Radioactivity (cpm/ml) of each sample was measured, and SDS-PAGE was performed to determine the fraction of radioactivity associated with whole immunoconjugate. Immunoconjugate-associated serum radioactivity was analyzed using the computer program PCNONLIN (SCI Software, Lexington, Kentucky). Table 7 below lists the pharmacokinetic parameters of the immunoconjugates. In that table, the standard error for each value is indicated and a one way analysis of variance is presented, IC is the immunoconjugate (specified by the abbreviation for the

-78-

gelonin variant that is part of the immunoconjugate),  $n$  is the number of animals in the study,  $V_c$  is the central volume of distribution,  $Cl$  is the clearance,  $MRT$  is the total body mean residence time,  $\alpha$  is the  $\alpha$  half-life and  $\beta$  is the  $\beta$  half-life of the immunoconjugate.

5

Table 7

IC	Vc (ml/kg)	Cl (ml/hr/kg)	MRT (hours)	Alpha (hours)	Beta (hours)
H65 Gel <sub>C247</sub> n=32	65.3 ± 3.4	11.0 ± 0.4	16.5 ± 1.9	2.3 ± 0.2	20.5 ± 3.0
H65 Gel <sub>A50(C44)</sub> n=38	61.9 ± 2.4	4.1 ± 0.1	22.7 ± 0.7	3.0 ± 0.7	17.8 ± 0.8
H65 Gel <sub>C10</sub> n=45	59.2 ± 1.3	2.5 ± 0.04	42.7 ± 1.1	3.3 ± 0.3	32.9 ± 1.1
p-value	0.176	<0.0001	<0.0001	0.303	<0.0001

-80-

The Gel<sub>C247</sub> immunoconjugate was found to have  $\alpha$  and  $\beta$  half lives of 2.3 and 20 hours, with a total mean residence time of 17 hours. The 72 and 96 hour time points were excluded from analysis because of the poor resolution of immunoconjugate associated radioactivity on the SDS-PAGE gel for these serum samples.

Because *in vitro* studies suggested that the Gel<sub>C10</sub> immunoconjugate had greater disulfide bond stability, it was anticipated that its half lives *in vivo* would be longer relative to the cys<sub>247</sub> form of the immunoconjugate. The  $\beta$  half life of the immunoconjugate was about 33 hours compared to 20 hours for the Gel<sub>C247</sub> conjugate. The total mean residence time was also much greater for the Gel<sub>C10</sub> immunoconjugate (42 hours versus 42 hours for the Gel<sub>C247</sub> conjugate). In addition, the clearance of the Gel<sub>C10</sub> immunoconjugate was 2.5 ml/hr/kg, about four times less than that of the Gel<sub>C247</sub> immunoconjugate (11 ml/hr/kg). As also predicted from the *in vitro* disulfide stability data, the clearance of the Gel<sub>A50(C44)</sub> immunoconjugate was intermediate between those of the Gel<sub>C10</sub> and Gel<sub>C247</sub> immunoconjugates.

Based on these studies, the Gel<sub>C10</sub> analog conjugated to H65 antibody has greater *in vivo* stability than the Gel<sub>A50(C44)</sub> and Gel<sub>C247</sub> analogs conjugated to H65 antibody (as determined by the longer mean residence time and clearance rates), although the properties of the Gel<sub>A50(C44)</sub> immunoconjugate more closely resembled those of the Gel<sub>C10</sub> immunoconjugate than the Gel<sub>C247</sub> immunoconjugate.

#### Example 11

##### Pharmacokinetics Of Conjugates To H65 Antibody Fragments

The pharmacokinetics of Gel<sub>C247</sub> and Gel<sub>A50(C44)</sub> analogs linked to human engineered H65 Fab fragments were also investigated in rats. An IV bolus of 0.1 mg/kg of <sup>125</sup>I-labelled he1 H65 Fab-Gel<sub>C247</sub>, he2 H65 Fab-Gel<sub>A50(C44)</sub>, or he3 H65 Fab-Gel<sub>A50(C44)</sub>, was administered to male Sprague-Dawley rats weighing 150-180 grams. Serum samples were collected at 3,

-81-

5, 15, 20, 30, and 40 minutes, and 1, 1.5, 3, 6, 8, 18, 24, 32, 48, and 72 hours, and were analyzed by ELISA using rabbit anti-Gelonin antibody as the capture antibody and biotin-labelled goat anti-human kappa light chain antibody as the secondary antibody. Results of the analysis are presented in Table 8 below. In the table, the standard error for each value is shown, and IC is the immunoconjugate, n is the number of animals in the study, Vc is the central volume of distribution, Vss is the steady state volume of distribution, Cl is the clearance, MRT is the total body mean residence time, Alpha is the  $\alpha$  half-life and Beta is the  $\beta$  half-life of the indicated conjugate.

Table 8

<u>IC</u>	<u>Vc</u> (ml/kg)	<u>Vss</u> (ml/hr/kg)	<u>Cl</u> (ml/hr/kg)	<u>MRT</u> (hours)	<u>Alpha</u> (hours)	<u>Beta</u> (hours)
he1 Gel <sub>C247</sub> n=27	48 ± 3	133 ± 7	62 ± 3	2.1 ± 0.1	0.33 ± 0.03	3.0 fixed
he2 Gel <sub>A50(C44)</sub> n=28	54 ± 5	141 ± 8	53 ± 3	2.7 ± 0.2	0.37 ± 0.04	3.1 fixed
he3 Gel <sub>A50(C44)</sub> n=33	77 ± 6	140 ± 20	57 ± 3	2.5 ± 0.4	0.58 ± 0.11	3.0 ± 1.0



-83-

Comparing the three immunoconjugates, the pharmacokinetics of he1 H65 Fab-Gel<sub>C247</sub>, he2 H65 Fab-Gel<sub>A50(C44)</sub> and he3-Fab-Gel<sub>A50(C44)</sub> were very similar, having similar alpha and beta half-lives, mean residence times, and clearance, particularly when comparing parameters obtained from the ELISA assayed curves. This is in contrast to their whole antibody immunoconjugate counterparts, where the clearance of Gel<sub>C247</sub> immunoconjugate (11 ml/kg/hr) was three-fold greater than that of Gel<sub>A50(C44)</sub> immunoconjugate (4 ml/kg/hr). This suggests that cleavage of the disulfide bond linking the Fab fragment and gelonin is not as important for the serum clearance of Fab immunoconjugates as for whole antibody immunoconjugates.

#### Example 12

##### Immunogenicity Of Immunoconjugates

Outbred Swiss/Webster mice were injected repeatedly (0.2 mg/kg each injection) with murine H65 antibody conjugates prepared with RTA, RTA30 and recombinant gelonin. The cycle was such that each animal was injected on days 1 and 2, and then the injections were repeated 28 and 29 days later. The animals received 5 such cycles of injections. One week and three weeks following each series of injections, blood was collected and the amount of anti-RIP antibodies present was determined by ELISA; peak titers for each cycle are shown in Table 9. RTA and RTA30 generated strong responses which began immediately following the first cycle of injections and remained high throughout the experiment. In contrast, no immune response was detected for the gelonin conjugate, even after 5 cycles of injections. When the conjugates were mixed with Complete Freund Adjuvant and injected i.p. into mice, anti-RTA and RTA-30 antibodies were readily detected after several weeks. These data indicate that anti-gelonin antibodies, if generated, would have been detected by the ELISA assay, and suggest that recombinant

-84-

gelonin may be much less immunogenic in animals than is RTA.

Table 9

	<u>Cycle</u>	<u>H65-RTA</u>	<u>H65-RTA30</u>	<u>H65-rGel</u>
5	Prebleed	100	100	100
	Cycle 1	168	117	100
	Cycle 2	4208	1008	100
	Cycle 3	7468	3586	100
	Cycle 4	5707	3936	100
10	Cycle 5	4042	2505	100

Example 13In vivo Efficacy Of Immunoconjugates

A human peripheral blood lymphocyte (PBL)-reconstituted, severe combined immunodeficient mouse model was utilized to evaluate the *in vivo* efficacy of various immunoconjugates comprising the gelonin analogs Gel<sub>C247</sub> and Gel<sub>A50(C44)</sub>. Immunoconjugates were tested for the capacity to deplete human blood cells expressing the CD5 antigen.

A. Human PBL Donors And Cell Isolation

Human peripheral blood cells were obtained from lymphapheresis samples (HemaCare Corporation, Sherman Oaks, CA) or venous blood samples (Stanford University Blood Bank, Palo Alto, CA) collected from healthy donors. Blood cells were enriched for PBLs using Ficoll-Hypaque density gradient centrifugation (Ficoll-Paque®; Pharmacia, Piscataway, New Jersey) and subsequently washed 4 times with PBS. Residual erythrocytes were lysed with RBC lysing buffer (16  $\mu$ M ammonium chloride, 1 mM potassium bicarbonate, 12.5  $\mu$ M EDTA) during the second wash. Cell viability in the final suspension was >95% as assessed by trypan blue dye exclusion.

-85-

### B. Animals And Human PBL Transfer

CB.17 scid/scid (SCID) mice were purchased from Taconic (Germantown, New York) or were bred under sterile conditions in a specific pathogen-free animal facility (original breeding pairs were obtained from Hana Biologics, Alameda, California). Animals were housed in filter-top cages and were not administered prophylactic antibiotic treatment. Cages, bedding, food and water were autoclaved before use. All manipulations with animals were performed in a laminar flow hood.

Untreated SCID mice were bled for determination of mouse Ig levels. Human PBL-injected mice were bled at various intervals for quantitation of human Ig and sIL-2R. Blood collection was from the retro-orbital sinus into heparinized tubes. Blood samples were centrifuged at 300 x g for 10 min, and plasma was collected and stored at -70°C. Mouse and human Ig were quantified using standard sandwich ELISAs. Briefly, flat-bottom microtiter plates (MaxiSorp Immuno-Plates, Nunc, Roskilde, Denmark) were coated overnight at 4°C with goat anti-mouse IgG+IgA+IgM (Zymed Laboratories, Inc., South San Francisco, California) or goat anti-human Igs (Tago, Inc., Burlingame, California) in bicarbonate buffer, pH 9.6. Plates were blocked for 2 hours at room temperature with 1% BSA in Tris-buffered saline, pH 7.5 (TBS), and then incubated at 37°C for 1 hour with standards or samples serially-diluted in TBS/1% BSA/0.05% Tween 20. Standards used were a monoclonal mouse IgG2a (IND1 anti-melanoma; XOMA Corporation, Berkeley, California) and polyclonal human Ig (Sigma Chemical Co., St. Louis, Missouri). Subsequently, plates were washed with TBS/Tween 20 and incubated at 37°C for 1 hour with alkaline phosphatase-conjugated goat anti-mouse IgG+IgA+IgM or goat anti-human Igs (Caltag Laboratories, South San Francisco, California). Detection was by measurement of absorbance at 405 nm following incubation with 1 mg/ml p-nitro-phenylphosphate (Sigma) in 10% diethanolamine buffer, pH 9.8. Plasma from a normal BALB/c mouse was used as a

-86-

positive control in the mouse Ig ELISA. Plasma samples from naive SCID mice or normal BALB/c mice did not have detectable levels of human Ig. Human sIL-2R was quantified using an ELISA kit (Immunotech S.A., Marseille, France) as per the manufacturer's instructions.

Five-to-seven week old mice with low plasma levels of mouse Ig ( $<10\mu\text{g/ml}$ ) were preconditioned with an i.p. injection of cyclophosphamide (Sigma) at 200 mg/kg. Two days later, they were injected i.p. with  $25-40 \times 10^6$  freshly-isolated human PBL suspended in 0.8 ml PBS.

#### C. Immunoconjugate Treatment

SCID mice were bled at approximately 2 weeks after human PBL transplantation. Mice with undetectable ( $<10 \text{ pM}$ ) or low plasma levels of human sIL-2R were eliminated from the study. The cut-off for exclusion of mice with detectable, but low, levels of human sIL-2R was empirically determined for each study and was generally 20 pM. The remaining mice were divided into groups and were administered vehicle or immunoconjugate as an i.v. bolus (0.2 mg/kg) daily for 5 consecutive days. Animals were sacrificed 1 day after cessation of treatment for quantitation of human T cells in tissues and human sIL-2R in plasma.

#### D. Collection Of Tissues And Analysis Of PBL Depletion

Blood was collected from the retro-orbital sinus into heparinized tubes. Mice were then killed by cervical dislocation and spleens were removed aseptically. Single cell suspensions of splenocytes were prepared in HBSS by pressing the spleens between the frosted ends of sterile glass microscope slides. Collected cells were washed twice with PBS. Erythrocytes were eliminated from blood and splenocyte suspensions using RBC lysing buffer. Subsequently, cells were resuspended in PBS for enumeration. Recovered cells were then assayed for Ag expression using flow cytometry.

-87-

Two to five hundred thousand cells in 100  $\mu$ l of PBS/1% BSA/0.1% sodium azide were incubated on ice for 30 min. with saturating amounts of various FITC- or phycoerythrin (PE)-conjugated Abs (Becton-Dickinson, Mountain View, CA) Abs used for staining included: HLe-1-FITC (IgG1 anti-CD45), Leu 2-FITC (IgG1 anti-CD8), Leu 3 PE (IgG1 anti-CD4), and Leu M3-PE (IgG2a anti-CD14). Cells were then washed in cold buffer and fixed in 0.37% formaldehyde in PBS. Samples were analyzed on a FACscan (Becton-Dickinson) using log amplifiers. Regions to quantify positive cells were set based on staining of cells obtained from naive SCID mice. The absolute numbers of human Ag-positive cells recovered from SCID tissues were determined by multiplying the percent positive cells by the total number of cells recovered from each tissue sample. The total number of leukocytes in blood was calculated using a theoretical blood volume of 1.4 ml/mouse. The detection limit for accurate quantitation of human cells in SCID mouse tissues was 0.05%. All statistical comparison between treatment groups were made using the Mann-Whitney U test. Treatment groups were determined to be significantly different from buffer control groups when the p value was  $<0.05$ . Results are presented in Table 10 below, wherein + indicates a significant difference from controls, - indicates an insignificant difference and NT means the conjugate was not tested. CD5 Plus (XOMA Corporation, Berkeley, California) is mouse H65 antibody chemically linked to RTA and is a positive control. OX19 Fab-Gel<sub>C247</sub> is a negative control immunoconjugate. The OX19 antibody (European Collection of Animal Cell Cultures #84112012) is a mouse anti-rat CD5 antibody that does not cross react with human CD5.

-88-

Table 10

<u>Test Article</u>		<u>Human T Cell Depletion</u>	
		<u>Spleen</u>	<u>Blood</u>
5	CD5 Plus	+	+
	cH65 F(ab') <sub>2</sub>	-	-
	cH65 Fab'	-	-
	H65-rGEL	+	+
	cH65 F(ab') <sub>2</sub> -rGel	+	+
10	cH65 Fab'-rGel	+	+
	cH65 F(ab') <sub>2</sub> -Gel <sub>c247</sub>	+	NT
	cH65 Fab'-Gel <sub>c247</sub>	+	+
	helH65 Fab'-Gel <sub>c247</sub>	+	NT
	cH65 Fab'-Gel <sub>AS0(C44)</sub>	+	+
15	OX19 Fab-Gel <sub>c247</sub>	-	-
	All the gelonin immunoconjugates were capable of depleting human cells in the SCID mouse model.		

Example 14

Construction Of Gelonin  
Immunofusions With Chimeric Antibodies

20 Several genetic constructs were assembled which included a natural sequence gelonin gene fused to an H65 truncated heavy chain gene (Fd or Fd'), or an H65 light chain gene (kappa). In this Example, H65 Fd, Fd', and H65 light chain refer to chimeric constructs. The H65 Fd

25 sequence consists of the nucleotides encoding the murine H65 heavy chain variable (V), joining (J) and human IgG<sub>1</sub>, constant (C) domain 1 regions, including the cysteine bound to light chain IgG<sub>1</sub> and has the carboxyl terminal sequence SCDKTHT (SEQ ID NO: 130). The H65 Fd' sequence has the H65

30 Fd sequence with the addition of the residues CPP from the hinge region of human IgG<sub>1</sub> heavy chain, including a cysteine residue which is bound to the other human IgG<sub>1</sub> heavy chain and its F(ab')<sub>2</sub> fragment. See Better, et al., Proc. Nat. Acad. Sci. (USA), 90: 457-461 (1993), incorporated by

35 reference herein.

-89-

The H65 light chain sequence consists of the nucleotides encoding the murine H65 light chain variable (V), joining (J), and human kappa ( $C_k$ ) regions. The DNA sequences of the V and J regions of the H65 Fd and kappa fragment genes linked to the *pelB* leader can be obtained from GenBank (Los Alamos National Laboratories, Los Alamos, New Mexico) under Accession Nos. M90468 and M90467, respectively. Several of the gene fusions included a gelonin gene linked at the 5' end of an H65 Fab fragment gene while the others included a gelonin gene linked at the 3' end of an H65 Fab fragment gene. A DNA linker encoding a peptide segment of the *E. coli* shiga-like toxin (SLT) (SEQ ID NO: 56), which contains two cysteine residues participating in a disulfide bond and forming a loop that includes a protease sensitive amino acid sequence) or of rabbit muscle aldolase [(RMA) as in SEQ ID NO: 57, which contains several potential cathepsin cleavage sites] was inserted between the gelonin gene and the antibody gene in the constructs. Alternatively, a direct fusion was made between a gelonin gene and an H65 Fab fragment gene without a peptide linker segment. Table 11 below sets out a descriptive name of each gene fusion and indicates the expression plasmid containing the gene fusion and the section of the application in which each is designated. Each plasmid also includes the Fab fragment gene (shown in parentheses in Table 11) with which each particular gene fusion was co-expressed. The inclusion of a cysteine from a hinge region (Fd') allows potential formation of either monovalent Fab' or bivalent F(ab')<sub>2</sub> forms of the expression product of the gene fusion.

-90-

Table 11

	<u>Section</u>	<u>Plasmid</u>	<u>Description</u>
	B(i)	pING3754	Gelonin::SLT::Fd' (kappa)
	B(ii)	pING3757	Gelonin::SLT::kappa (Fd')
5	B(iii)	pING3759	Gelonin::RMA::Fd' (kappa)
	B(iv)	pING3758	Gelonin::RMA::kappa (Fd')
	A(i)	pING4406	Fd::SLT::Gelonin (kappa)
	A(ii)	pING4407	kappa::SLT::Gelonin (Fd)
	A(iii)	pING4408	Fd::RMA::Gelonin (kappa)
10	A(iv)	pING4410	kappa::RMA::Gelonin (Fd)
	C(i)	pING3334	Gelonin::Fd (kappa)

A. Fusions Of Gelonin At The  
Carboxyl-Terminus Of Antibody Genes

(i) Fd::SLT::Gelonin (kappa)

15 A gelonin gene fusion to the 3'-end of the H65 Fd chain with the 23 amino acid SLT linker sequence was assembled in a three piece ligation from plasmids pVK1, pING3731 (ATCC 68721) and pING4000. Plasmid pVK1 contains the Fd gene linked in-frame to the SLT linker sequence and  
20 some H65 Fd' and kappa gene modules as in pING3217, shown in Better, et al., *Proc. Nat. Acad. Sci. (USA)*: 457-461 (1993), except that the kappa and Fd' regions are reversed. Plasmid pING3731 contains the gelonin gene, and pING4000 contains the H65 kappa and Fd' genes each linked to the  
25 pelB leader sequence under the control of the araB promoter as a dicistronic message.

30 Plasmid pVK1 was designed to link the 3'-end of a human IgG Fd constant region in-frame to a protease-sensitive segment of the SLT gene bounded by two cysteine residues which form an intra-chain disulfide bond. The SLT gene segment (20 amino acids from SLT bounded by cysteine residues, plus three amino acids introduced to facilitate cloning) was assembled from two oligonucleotides, SLT Linker 1 and SLT Linker 2.



-91-

SLT Linker 1 (SEQ ID NO: 73)

5' TGTCATCATCATGCATCGCGAGTTGCCAGAATGGCATCT  
GATGAGTTTCCTTCTATGTGCGCAAGTACTC 3'

SLT Linker 2 (SEQ ID NO: 74)

5' TCGAGAGTACTTGGCGCACATAGAAGGAAACTCATCAGAT  
GCCATTCTGGCAACTCGCGATGCATGATGACATGCA 3'

The two oligonucleotides were annealed and ligated into a vector (pING3185) containing *Pst*I and *Xho*I cohesive ends, destroying the *Pst*I site and maintaining the *Xho*I site.

10 Plasmid pING3185 contained an engineered *Pst*I site at the 3'-end of the Fd gene, and contained an *Xho*I site downstream of the Fd gene. The product of this ligation, pVK1, contained the H65 Fd gene (fused to the pelB leader) in frame with the SLT linker segment, and contained two  
15 restriction sites, *Fsp*I and *Sca*I, at the 3'-end of the SLT linker.

Plasmid pVK1 was digested with *Sau*I and *Sca*I, and the 217 bp fragment containing a portion of the Fd constant domain and the entire SLT gene segment was purified by  
20 electrophoresis on an agarose gel. pING3731 was digested with *Sma*I and *Xho*I and the 760 bp gelonin gene was similarly purified. Plasmid pING4000 was digested with *Sau*I and *Xho*I and the vector segment containing the entire kappa gene and a portion of the Fd gene was also purified.  
25 Ligation of these three DNA fragments resulted in pING4406 containing the Fd::SLT::Gelonin (kappa) gene fusion vector.

(ii) kappa::SLT::Gelonin (Fd)

A gelonin gene fusion to the 3'-end of the H65 kappa chain with the 25 amino acid SLT linker sequence (20  
30 amino acids from SLT bounded by cysteine residues, plus 5 amino acids introduced to facilitate cloning) was assembled from the DNA segments in pING3731 (ATCC 68721) and pING3713.

Plasmid pING3713 is an Fab expression vector  
35 where the H65 Fd and kappa genes are linked in a dicistronic transcription unit containing the SLT linker

-92-

segment cloned in-frame at the 3'-end of the kappa gene. The plasmid was constructed as follows. In a source plasmid containing the H65 Fd and kappa genes, an *EagI* site was positioned at the 3'-end of the kappa gene by site directed mutagenesis without altering the encoded amino acid sequence. The SLT gene segment from pVK1 was amplified with primers SLT-*EagI*-5' and *SalI* for in frame linkage to the *EagI* site at the 3'-end of the kappa gene.

SLT-*EagI*-5' (SEQ ID NO: 75)

5' TGTTCGGCCGCATGTCATCATCATGCATCG 3'

*SalI* (SEQ ID NO: 76)

5' AGTCATGCCCCGCGC 3'

The 140 bp PCR product was digested with *EagI* and *XhoI*, and the 75 bp fragment containing the SLT gene segment was cloned adjacent to the Fd and kappa genes in the source plasmid to generate pING3713.

For construction of gene fusion to gelonin, pING3713 was cut with *ScaI* and *XhoI*, and the vector fragment containing the Fd gene and kappa::SLT fusion was purified. pING3731 was digested with *SmaI* and *XhoI* and the DNA fragment containing the gelonin gene was also purified. The product of the ligation of these two fragments, pING4407, contains the Fd and kappa::SLT::gelonin genes.

(iii) Fd::RMA::Gelonin (kappa)

A gelonin gene fusion to the 3'-end of the H65 Fd chain with the 21 amino acid RMA linker sequence (20 amino acids from RMA, plus 1 amino acid introduced to facilitate cloning) was assembled in a three piece ligation from plasmids pSH4, pING3731 (ATCC 68721) and pING4000.

Plasmid pSH4 contains an Fd gene linked in frame to the RMA linker sequence. The RMA gene segment was linked to the 3'-end of Fd by overlap extension PCR as follows. The 3'-end (constant region) of the Fd gene was amplified by PCR from a source plasmid with the primers KBA- $\gamma$ 2 and RMAG-1. Any Fd constant region may be used

-93-

because constant regions of all human IgG<sub>1</sub> antibodies are identical in this region.

KBA-γ2 (SEQ ID NO: 77)

5' TCCCGGCTGTCCTACAGT 3'

5

RMAG-1 (SEQ ID NO: 78)

5' TCCAGCCTGTCCAGATGGTGTGTGAGTTTTGTCACAA 3'

The product of this reaction was mixed with primer RMA-76, which annealed to the amplified product of the first reaction, and the mixture was amplified with primers KBA-γ2

10 and RMAK-2.

RMA-76 (SEQ ID NO: 79)

5' CTAAGTCGAGAGTACTGTATGCATGGTTCGAGATGAACA  
AAGATTCTGAGGCTGCAGCTCCAGCCTGTCCAGATGG 3'

RMAK-2 (SEQ ID NO: 80)

15

5' CTAAGTCGAGAGTACTGTAT 3'

The PCR product contained a portion of the Fd constant region linked in-frame to the RMA gene segment. The product also contained a ScaI restriction site useful for in-frame fusion to a protein such as gelonin, and an XhoI site for subsequent cloning.

20

This PCR product was cut with SauI and XhoI and ligated adjacent to the remainder of the Fd gene to generate pSH4.

For assembly of the gene fusion vector containing the Fd::RMA::Gelolin, kappa genes, pSH4 was cut with SauI and ScaI and the Fd::RMA segment was purified. Plasmid pING3731 was cut with SmaI and XhoI and the 760 bp DNA fragment containing the gelonin gene was purified, and pING4000 was cut with SauI and XhoI and the vector was purified. The product of the ligation of these fragments, pING4408, contained the Fd::RMA::Gelolin and kappa genes.

30

(iv) kappa::RMA::Gelolin (Fd)

A gelonin gene fusion to the 3'-end of the H65 kappa chain with the 21 amino acid RMA linker sequence was assembled in a three piece ligation from plasmids pSH6, pING4408 (see the foregoing paragraph) and pING3713.

35

-94-

Plasmid pSH6 contains a kappa gene linked in-frame to the RMA linker sequence. The RMA gene segment was linked to the 3'-end of kappa by overlap extension PCR as follows. The 3'-end (constant region) of the kappa gene was amplified by PCR from a source plasmid with the primers KBA-K2 and RMAK-1.

RMAK-1 (SEQ ID NO: 81)

5' TCCAGCCTGTCCAGATGGACACTCTCCCCTGTTGAA 3'

KBA-K2 (SEQ ID NO: 82)

5' GTACAGTGAAGGTGGAT 3'

The product of this reaction was mixed with primer RMA-76 (SEQ ID NO: 81), which annealed to the amplified product of the first reaction, and the mixture was amplified with primers KBA-K2 and RMAK-2. The PCR product contained a portion of the kappa constant region linked in-frame to the RMA gene segment. The product also contained a *ScaI* restriction site useful for in-frame fusion to a protein such as gelonin, and an *XhoI* site for subsequent cloning. This PCR product was cut with *SstI* and *XhoI* and ligated adjacent to the remainder of the kappa gene to generate pSH6.

For assembly of the gene fusion vector containing the kappa::RMA::Gelonin and Fd genes, pSH6 was cut with *HindIII* and *PstI* and the DNA fragment containing the kappa constant region and a portion of the RMA linker (the *PstI* RMA linker segment contains a *PstI* site) segment was purified. Plasmid pING4408 was cut with *PstI* and *SalI* and the DNA fragment containing a segment of the RMA linker, the gelonin gene and a portion of the tetracycline resistance gene in the vector segment was purified. pING3713 was cut with *SalI* and *HindIII* and the vector was purified. The product of the ligation of these three fragments, pING4410, contained the kappa::RMA::Gelonin and Fd genes.

-95-

B. Fusions Of Gelonin At The  
Amino-Terminus Of Antibody Genes

(i) Gelonin::SLT::Fd' (kappa)

A gelonin gene fusion to the 5'-end of the H65  
 5 Fd' chain with a 25 amino acid SLT linker sequence (20  
 amino acids from SLT bounded by cysteine residues, plus  
 five amino acids introduced to facilitate cloning) was  
 assembled in a three piece ligation from plasmids pING3748,  
 pING3217, and a PCR fragment encoding the H65 heavy chain  
 10 variable region ( $V_H$ ) gene segment which is the variable  
 region of the Fd' gene in pING3217. Plasmid pING3748  
 contains the gelonin gene linked in-frame to the SLT linker  
 sequence, and pING3217 contains the H65 Fd' and kappa genes  
 in a dicistronic transcription unit.

15 Plasmid pING3825 (see Example 2) was amplified  
 with PCR primers gelo3'-Eag and gelo-9 to introduce an EagI  
 restriction site at the 3'-end of the gelonin gene by PCR  
 mutagenesis.

gelo3'-Eag (SEQ ID NO: 83)

20 5' CATGCGGCCGATTTAGGATCTTTATCGACGA 3'

The PCR product was cut with BclI and EagI and the 56 bp  
 DNA fragment was purified. Plasmid pING3713 was cut with  
 EagI and XhoI, and the 77 bp DNA fragment containing the  
 SLT linker was purified. The 56 bp BclI to EagI fragment  
 25 and the 77 bp EagI to XhoI fragment were ligated into  
 pING3825 which had been digested with BclI and XhoI to  
 generate pING3748 which contains the gelonin gene linked  
 in-frame to the SLT linker sequence.

For assembly of the gene fusion vector containing  
 30 the Gelonin::SLT::Fd' and kappa genes, the H65  $V_H$  was  
 amplified by PCR from pING3217 with primers H65-G1 and H65-  
 G2, and the product was treated with T4 polymerase followed  
 by digestion with NdeI.

H65-G1 (SEQ ID NO: 84)

35 5' AACATCCAGTTGGTGCAGTCTG 3'

H65-G2 (SEQ ID NO: 85)

5' GAGGAGACGGTGACCGTGGT 3'

-96-

The 176 bp fragment containing the 5'-end of the H65 heavy chain V-region was purified. Concurrently, pING3217 was digested with *NdeI* and *XhoI*, and the 1307 bp DNA fragment containing a portion of the Fd' gene and all of the kappa gene was purified. The two fragments were ligated to pING3748 which had been digested with *ScaI* and *XhoI* in a three piece ligation yielding pING3754 (ATCC 69102), which contains the Gelonin::SLT::Fd' and kappa genes.

(ii) Gelonin::SLT::kappa (Fd')

A gelonin gene fusion to the 5'-end of the H65 kappa chain with the 25 amino acid SLT linker sequence was assembled in a three piece ligation from plasmids pING3748 (see the foregoing section), pING4000, and a PCR fragment encoding the H65 light chain variable region (V<sub>L</sub>) gene segment.

For assembly of the gene fusion vector containing the Gelonin::SLT::kappa and Fd' genes, an H65 V<sub>L</sub> fragment was amplified by PCR from pING3217 with primers H65-K1 and JK1-*HindIII*, and the product was treated with T4 polymerase followed by digestion with *HindIII*.

H65-K1 (SEQ ID NO: 86)

5' GACATCAAGATGACCCAGT 3'

JK1-*HindIII* (SEQ ID NO: 87)

5' GTTTGATTTCAAGCTTGGTGC 3'

The 306 bp fragment containing the light chain V-region was purified. Concurrently, pING4000 was digested with *HindIII* and *XhoI*, and the 1179 bp DNA fragment containing the kappa constant region and all of the Fd' gene was purified. The two fragments were ligated to pING3748 which had been digested with *ScaI* and *XhoI* in a three piece ligation yielding pING3757, which contains the Gelonin::SLT::kappa and Fd genes.

-97-

(iii). Gelonin::RMA::Fd' (kappa)

A gelonin gene fusion to the 5'-end of the H65 Fd' chain with the 24 amino acid RMA linker sequence (20 amino acids from RMA, plus 4 amino acids introduced to facilitate cloning) was assembled in a three piece ligation from plasmids pING3755, pING3217 and a PCR fragment encoding the H65 V<sub>H</sub> gene segment. Plasmid pING3755 contains the gelonin gene linked in-frame to the RMA linker sequence, and pING3217 contains the H65 Fd' and kappa genes in a dicistronic transcription unit.

Plasmid pING3755 was assembled to contain the gelonin gene linked to the RMA linker gene segment. The RMA linker gene segment was amplified by PCR from pSH4 with primers RMA-EagI and HINDIII-2.

RMA-EagI (SEQ ID NO: 88)  
5' ACTTCGGCCGCACCATCTGGACAGGCTGGAG 3'  
HINDIII-2 (SEQ ID NO: 44)  
5' CGTTAGCAATTCTAACTGTGAT 3'

The 198 bp PCR product was cut with *EagI* and *HindIII*, and the resulting 153 bp DNA fragment was purified. This RMA gene segment was cloned adjacent to gelonin using an *PstI* to *EagI* fragment from pING3748 and the *PstI* to *HindIII* vector fragment from pING3825. The product of this three piece ligation was pING3755.

For assembly of the gene fusion vector containing the Gelonin::RMA::Fd', kappa genes, the H65 V<sub>H</sub> was amplified by PCR from pING3217 with primers H65-G1 (SEQ ID NO: 84) and H65-G2 (SEQ ID NO: 85), and the product was treated with T4 polymerase followed by digestion with *NdeI*. The 186 bp fragment containing the 5'-end of the heavy chain V-region was purified. Concurrently, pING3217 was digested with *NdeI* and *XhoI*, and the 1307 bp DNA fragment containing a portion of the Fd' gene and all of the kappa gene was purified. These two fragments were ligated to pING3755 which had been digested with *ScaI* and *XhoI* in a three piece ligation yielding pING3759 (ATCC 69104), which contains the Gelonin::RMA::Fd' and kappa genes.

-98-

(iv) Gelonin::RMA::kappa (Fd')

A gelonin gene fusion to the 5'-end of the H65 kappa chain with the 24 amino acid RMA linker sequence was assembled in a three piece ligation from plasmids pING3755, pING4000, and a PCR fragment encoding the H65 V<sub>L</sub> gene segment.

For assembly of the gene fusion vector containing the Gelonin::RMA::kappa and Fd' genes, an H65 V<sub>L</sub> segment was amplified by PCR from pING3217 with primers H65K-1 (SEQ ID NO: 86) and JK1-HindIII, and the product was treated with T4 polymerase followed by digestion with HindIII. The 306 bp fragment containing the 5'-end of the light chain V-region was purified. Concurrently, pING4000 was digested with HindIII and XhoI, and the 1179 bp DNA fragment containing the kappa constant region and all of the Fd' gene was purified. These two fragments were ligated to pING3755 which had been digested with ScaI and XhoI in a three piece ligation yielding pING3758 (ATCC 69103), which contains the Gelonin::RMA::kappa and Fd' genes.

C. Direct Fusions Of Gelonin At The Amino Terminus Of Antibody Genes

(i) Gelonin::Fd' (Kappa)

A direct gelonin gene fusion was constructed from pING3754. pING3754 was digested with BglIII and XhoI and the vector segment was purified. Concurrently, pING3754 was digested with EagI, treated with T4 polymerase, cut with BglIII, and the gelonin gene segment was purified. pING3754 was also cut with FspI and XhoI, and the Fd and kappa gene segment was purified. These fragments were assembled in a three-piece ligation to generate pING3334, which contains a direct gene fusion of gelonin to Fd' in association with a kappa gene.



-99-

Example 15Preparation of he3 Fab And Gelonin he3Fab Immunofusions

The sections below detail the construction of human-engineering he3Fab protein and immunofusions of gelonin to he3 Fd and kappa chains.

A. he3-Fab Expression Plasmids

The he3 heavy chain V-region was PCR-amplified from plasmid pING4621 (pING4621 is fully described above in Example 5 above), with primers H65-G3, GAGATCCAGTTGGTGCAGTCTG (SEQ ID NO: 116) and H65G2 (SEQ ID NO: 85). Amplification was carried at using vent polymerase (New England Biolabs) for 25 cycles, including a 94°C denaturation for 1 minute, annealing at 50°C for 2 minutes, and polymerization for 3 minutes at 72°C. The PCR product was treated with polynucleotide kinase and digested with BstEII and the V-region DNA was purified. The purified DNA fragment was then ligated into pIC100, which had been digested with SstI, treated with T4 polymerase, and cut with BstEII. The resulting fragment was then ligated with the BstEII fragment from pING3218 (containing Fab' genes) to make pING4623 which contained the he3 Fd gene linked to the pelB leader sequence.

The he3 kappa V-region was next assembled as described above in Example 5 and in co-owned, co-pending U.S. Patent Application Serial No. 07/808,464, incorporated by reference herein, using six oligonucleotide primers,

\$H65k-1, AGT CGT CGA CAC GAT GGA CAT GAG GAC CCC TGC TCA GTT TCT TGG CAT CCT CCT ACT CTG GTT TCC AGG TAT CAA ATG TGA CAT CCA GAT GAC TCA GT (SEQ ID NO: 117);

HUH-K6, TCA CTT GCC GGG CGA ATC AGG ACA TTA ATA GCT ATT TAA GCT GGT TCC AGC AGA AAC CAG GGA AAG CTC CTA AGA CCC T (SEQ ID NO: 118);

HUH-K7, TGA CTC GCC CGG CAA GTG ATA GTG ACT CTG TCT CCT ACA GAT GCA GAC AGG GAA GAT GGA GAC TGA GTC ATC TGG ATG TC (SEQ ID NO: 119);

-100-

HUH-K8, GAT CCA CTG CCA CTG AAC CTT GAT GGG ACC  
CCA GAT TCC AAT CTG TTT GCA CGA TAG ATC AGG GTC TTA GGA GCT  
TTC C (SEQ-ID-NO:-120);

5 HUH-K4, GGT TCA GTG GCA GTG GAT CTG GGA CAG ATT  
ATA CTC TCA CCA TCA GCA GCC TGC AAT ATG AAG ATT TTG GAA TTT  
ATT ATT G (SEQ ID NO: 121); and

HUH-K5, GTT TGA TTT CAA GCT TGG TGC CTC CAC CGA  
ACG TCC ACG GAG ACT CAT CAT ACT GTT GAC AAT AAT AAA TTC CAA  
AAT CTT C (SEQ ID NO: 122)  
10 and amplified with primers HUK-7 (SEQ ID NO: 92) and JK1-  
*HindIII* (SEQ ID NO: 87).

The resulting PCR product was treated with T4  
polymerase, digested with *HindIII*, and purified. The  
purified fragment was then cloned into pIC100, which had  
15 first been cut with *SstI*, treated with T4 polymerase, and  
digested with *XhoI*, along with the 353 bp *HindIII-XhoI*  
fragment encoding the kappa constant region from pING3217.  
The resulting plasmid was pING4627 which contains the he3  
kappa sequence linked in frame to the *pelB* leader.

20 Plasmid pING4628, containing the *pelB*-linked he3  
kappa and *Fd* genes under transcriptional control of the  
*araB* promoter, was assembled from pING4623 and pING4627 as  
follows.

An expression vector for unrelated kappa and *Fd*  
25 genes, pNRX-2, was first cut with *SauI* and *EcoRI*, leaving  
a vector fragment which contains all the features relevant  
to plasmid replication, a tetracycline resistance marker,  
*araB* transcriptional control, and the 3' end of the *Fd*  
constant region. [Plasmid pNRX-2 comprises an *EcoRI* to  
30 *XhoI* DNA segment from pING 3104 (described in WO 90/02569,  
incorporated by reference herein). That segment contains  
the replication, resistance and transcription control  
features of pING3104 and is joined to an *XhoI* to *SauI* DNA  
segment from pING1444 (described in WO 89/00999,  
35 incorporated by reference herein) which contains the 3' end  
of an *Fd* constant region.] Next pING4623 was cut with  
*PstI*, treated with T4 polymerase, digested with *SauI* and

-101-

the *pelB::Fd* gene segment was then isolated. Plasmid pING4627 was cut with *XhoI*, treated with T4 polymerase, cut with *EcoRI* and ligated to the *pelB::Fd* gene segment and the pNRX-2 vector fragment to generate the he3-Fab expression vector pING4628. That plasmid contains two *XhoI* sites, one located between the kappa and Fd genes, and another 4 bp downstream of the termination codon for the Fd gene.

A vector, pING4633, which lacks the *XhoI* site between the kappa and Fd genes was constructed. To assemble pING4633, pING4623 was cut with *EcoRI*, treated with T4 polymerase, digested with *SauI*. The *pelB::kappa* gene segment was then isolated and purified. The pNRX-2 vector fragment and the *pelB::Fd* gene segment were then ligated to the purified *pelB::kappa* gene segment to form pING4633.

Both pING4633 and pING4628 are bacterial expression vectors for he3-Fab and each comprises the he3 Fd and Kappa genes which are expressed as a dicistronic message upon induction of the host cell with L-arabinose. Moreover, pING4628 contains two *XhoI* restriction sites, one located 4bp past the Fd termination codon and one in the intergenic region between the 3' end of the Kappa gene and the 5' end of the Fd gene. Plasmid pING4633 lacks the *XhoI* site in the intergenic region.

#### 25 B. Purification Of he3Fab

Plasmids pING4628 and pING4633 were transformed into *E. coli* E104. Bacterial cultures were induced with arabinose and cell-free supernatant comprising the he3Fab was concentrated and filtered into 20 mM HEPES, pH 6.8. The sample was then loaded onto a CM Sphradex column (2.5 x 3 cm), equilibrated in 20 mM HEPES, 1.5 mM NaCl, pH 6.8. The column was washed with the same buffer and eluted with 20 mM HEPES, 27 mM NaCl, pH 6.8. The eluate was split into 2 aliquots and each was loaded onto and eluted from a protein G (Bioprocessing) column (2.5 x 2.5 cm) separately. The protein G column was equilibrated in 20 mM HEPES, 75 mM

-102-

NaCl, pH 6.8 and the sample was eluted with 100 mM glycine, 100 mM NaCl, pH 3.0. The two eluates were combined and diluted two times with 20 mM HEPES, 3 M ammonium sulfate, pH 6.8. The diluted eluates were loaded onto phenyl  
5 sepharose high substitution Fast Flow (Pharmacia) column (2.5 x 3.3 cm), equilibrated in 20 mM HEPES, 1.5 M ammonium sulfate, pH 6.8. The column was then eluted with 20 mM HEPES, 0.6 M ammonium sulfate, pH 6.8.

C. Gelonin::RMA::he3Kappa, he3Fd Fusions

10 Other genetic constructs were assembled which included a natural sequence gelonin gene fused to an he3-Fab via a linker.

A fusion comprising Gelonin::RMA::he3Kappa, Fd was assembled from DNA from plasmids pING3755, pING4633,  
15 and pING4628. Both pING4633 and pING4628 were assembled in a series of steps whereby the he3 heavy and light V-regions were individually linked in-frame to the pelB leader. The heavy and light V-regions were then placed together in a dicistronic expression vector under the control of the araB  
20 promoter in a bacterial expression vector.

Assembly of the Gelonin::RMA::he3Kappa, he3Fd fusions was accomplished by constructing three DNA fragments from plasmids pING3755, pING4633, and pING4628. The first such fragment was made by digesting pING3755 with  
25 ScaI and XhoI which excises the 4bp between those sites. The resulting vector fragment was purified. The second fragment for use in constructing the above fusions was obtained from plasmid pING4633, which was cut with AseI (which cuts in V<sub>L</sub>) and XhoI and the resulting 1404 bp  
30 fragment, containing the 3' end segment of the Kappa and Fd genes, was purified. The third fragment, comprising the 5' end of the Kappa variable region coding sequence, was prepared from the PCR amplified V<sub>L</sub> gene contained in pING4628 using the oligonucleotide primers, Huk-7 and jk1-  
35 HindIII. The resulting 322 bp PCR-amplified V<sub>L</sub> fragment was treated with T4 polymerase, digested with AseI, and the 86

-103-

bp fragments containing the 5' end of V<sub>1</sub> was purified. The three fragment produced above were ligated together to form pING3764. The DNA sequence of the PCR amplified V-region was verified by direct DNA sequencing of pING3764.

5 D. Gelonin::SLT::he3Kappa, he3Fd Fusion

A Gelonin::SLT::he3Kappa, he3Fd fusion was constructed by ligating the pING4633 and pING4628 fragments described in section A immediately above with a fragment produced from pING3748 which contains Gelonin::SLT. The  
10 pING3748 fragment was produced using ScaI and XhoI as described immediately above for pING3755. The resulting vector was designated pING3763.

E. Construction of Expression Vector Containing Gelonin::SLT::he3Fd, he3Kappa Fusions

15 An expression vector containing the Gelonin::SLT::he3Fd, he3kappa fusion was constructed in two steps from DNA segments from plasmids pING3825, pING4628, pING4639, pING3217 [described in Better, et al., *Proc. Natl. Acad. Sci. (USA)*, 90:457-461 (1993), incorporated by  
20 reference herein], and pING4627. pING3825 was digested with NcoI and XhoI, generating a 654 bp fragment containing the 3' end of the gelonin gene and a fragment containing the 5' end of the gelonin gene which were purified. Next, pING4639 was digested with NcoI and NdeI and the 903 bp  
25 fragment containing the 3' end of the Gelonin gene, the SLT linker, and the 5' end of V<sub>8</sub> which resulted was purified. Finally, pING4628 was cut with NdeI and XhoI, resulting in a 523 bp fragment containing the 3' end of the Fd gene which was purified. The three fragments were then ligated  
30 to form plasmid pING3765 which contains a gene encoding a gelonin::SLT::he3Fd fusion.

Three vector fragments were used to assemble the final expression vector (containing the gelonin::SLT::he3Fd and he3 kappa segments). Plasmid pING3765 was digested  
35 with XhoI, treated with T4 polymerase, cut with NheI (which releases a 265 bp fragment encoding the tetracycline

-104-

resistent marker), and the resulting vector fragment was purified. Plasmid pING4627, which contains the he3Kappa gene ~~linked in-frame~~ to the pelB leader was used for the construction of pING4628. Plasmid pING4627 was cut with PstI, treated with T4 polymerase, and further digested with SstI. The resulting 726 bp fragment, containing the Kappa gene (except 40 bp at the 3' end) was purified. Plasmid pING3217 was then cut with SstI and NheI, resulting in a 318 bp fragment containing the 3' end of the Kappa gene and downstream portion, including a portion of the tetracycline resistance gene, which was purified. Ligation of the foregoing three fragments produced the final expression vector, pING3767.

F. Construction Of Expression Vector  
Containing Gelonin::RMA::he3Fd Fusions

Gelonin::RMA:he3Fd, he3Kappa fusion expression vectors was constructed in two steps from plasmids pING3825, pING4628, pING3217, and pING4627. The cloning scheme used was identical to that used to generate pING3767 except that pING4638 was substituted for pING4639. Plasmid pING4638 differs from pING4639 as described below in Example 16. The intermediate vector encoding the Gelonin::RMA::Fd fusion was designated pING3766 and the final expression vector was designated pING3768.

Example 16  
Gelonin-Single Chain Antibody Fusions

The natural sequence gelonin gene was also fused to a single chain form of the human engineered he3 H65 variable region. The gelonin gene was positioned at either the N-terminus or the C-terminus of the fusion gene and the SLT or RMA linker peptide was positioned between the gelonin and antibody domains to allow intracellular processing of the fusion protein with subsequent cytosolic release of gelonin.

-105-

A. Construction of Gel::RMA::SCA(V<sub>L</sub>-V<sub>H</sub>), Gel::SLT::SCA(V<sub>L</sub>-V<sub>H</sub>), Gel::RMA::SCA(V<sub>H</sub>-V<sub>L</sub>), and Gel::SLT::SCA(V<sub>H</sub>-V<sub>L</sub>)

A single chain antibody (SCA) form of the he3 H65 variable domain was assembled from previously constructed genes. This SCA segment consisted of the entire V and J region of the one chain (heavy or light) linked to the entire V and J segment of the other chain (heavy or light) via a 15 amino acid flexible peptide: [(Gly)<sub>4</sub>Ser]<sub>3</sub>. This peptide is identical to that described in Huston et al., *Proc. Natl. Acad. Sci. USA*, 85:5879-5883 (1988); Glockshuber et al., *Biochemistry*, 29:1362-1367 (1990); and Cheadle et al., *Molecular Immunol.*, 29:21-30 (1992). The SCA was assembled in two orientations: V-J<sub>kappa</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>gamma</sub> and V-J<sub>gamma</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>kappa</sub>. Each SCA segment was assembled and subsequently fused to gelonin.

For assembly of the SCA segment V-J<sub>kappa</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>gamma</sub>, primers HUK-7 and SCFV-1 were used to amplify a 352 bp DNA fragment containing the he3 V/J kappa sequences from pING4627 by PCR in a reaction containing 10 mM KCl, 20 mM TRIS pH 8.8, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2mM MgSO<sub>4</sub>, 0.1% Triton X-100., 100 ng/ml BSA, 200 uM of each dNTP, and 2 Units of Vent polymerase (New England Biolabs, Beverley, Massachusetts) in a total volume of 100 µl.

SCFV-1 (SEQ ID NO:91)  
 5' CGGACCCACCTCCACCAGATCCACCGC  
 CACCTTTCATCTCAAGCTTGGTGC 3'  
 HUK-7 (SEQ ID NO: 92)  
 5' GACATCCAGATGACTCAGT 3'

Concurrently, primers SCFV-2 and SCFV-3 were used to amplify a he3 heavy chain V/J gamma segment from pING4623, generating a 400 bp fragment.

-106-

SCFV-2 (SEQ ID NO: 93)

5' GGTGGAGGTGGGTCCGGAGGTGGAGGATCTGA

GATCCAGTTGGTGCACT 3'

SCFV-3 (SEQ ID NO: 94)

5 TGTACTCGAGCCCATCATGAGGAGACGGTGACCGT 3'

The products from these reactions were mixed and amplified with the outside primers HUK-7 and SCFV-3. The product of this reaction was treated with T4 polymerase and then cut with *Xho*I. The resulting 728 bp fragment was then purified by electrophoresis on an agarose gel. This fragment was ligated into the vectors pINC3755 and pINC3748 (see Example 10), each digested with *Sca*I and *Xho*I. The resulting vectors pINC4637 and pINC4412 contain the Gelonin::RMA::SCA V-J<sub>kappa</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>Gamma</sub> and Gelonin::SLT::SCA V-J<sub>kappa</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>Gamma</sub> fusion genes, respectively. The 728 bp fragment was also ligated into pIC100 previously digested with *Sst*I, treated with T4 polymerase and digested with *Xho*I, to generate pINC4635. This plasmid contains the *pelB* leader sequence linked in-frame to the V-J<sub>kappa</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>Gamma</sub> gene. The *pelB*::SCA gene in pINC4635 was excised as an *Eco*RI-*Xho*I restriction fragment and cloned into the bacterial expression vector to generate pINC4640.

Similarly, the SCA V-J<sub>Gamma</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>kappa</sub> was assembled by amplification of pINC4627 with primers SCFV-5 and SCFV-6 generating a 367 bp fragment containing he3 V/J kappa sequences,

SCFV-5 (SEQ ID NO: 95)

5' GGTGGAGGTGGGTCCGGAGGTGGAGGATCT  
GACATCCAGATGACTCAGT 3'

SCFV-6 (SEQ ID NO: 96)

5' TGTACTCGAGCCCATCATTTTCATCTCAAGCTTGGTG 3'

and pINC4623 with primers H65-G3 and SCFV-4 generating a 385 bp fragment containing he3 gamma V/J sequences by PCR with Vent polymerase.

H65-G3 (SEQ ID NO: 97)

5' GAGATCCAGTTGGTGCACTCTG 3'



-107-

SCFV-4 (SEQ ID NO: 98)

5' CGGACCCACCTCCACCAGATCC

ACCGCCACCTGAGGAGACGGTGACCGT 3'

The products from these reactions were mixed and amplified  
 5 with H65-G3 and SCFV-6. The 737 bp product was treated  
 with T4 polymerase and cut with *Xho*I. Ligation into  
 pING3755 and pING3748 (digested with *Sca*I and *Xho*I)  
 resulted in assembly of the Gelonin::RMA::SCA V-  
 J<sub>Gamma</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>Kappa</sub> gene fusion in pING4638 and  
 10 Gelonin::SLT::SCA V-J<sub>Gamma</sub>::[(Gly)<sub>4</sub>Ser]<sub>3</sub>::V-J<sub>Kappa</sub> gene fusion  
 in pING4639, respectively.

The vectors pING4637, pING4412, pING4638 and  
 pING4639 were each transformed into *E. coli* strain E104 and  
 induced with arabinose. Protein products of the predicted  
 15 molecular weight were identified by Western blot with  
 gelonin-specific antibodies.

#### B. Construction of

##### SCA(V<sub>L</sub>-V<sub>H</sub>)::SLT::Gelonin Vectors

The expression vector containing SCA(V<sub>L</sub>-  
 20 V<sub>H</sub>)::SLT::Gelonin fusions was assembled using restriction  
 fragments from previously-constructed plasmids pING4640  
 (containing SCA(V<sub>L</sub>-V<sub>H</sub>)) pING4407 (containing  
 Kappa::SLT::Gelonin, Fd), and pING3197. Plasmid pING4640  
 was first cut with *Bsp*HI, filled in with T4 polymerase in  
 25 the presence of only dCTP, treated with mung bean nuclease  
 (MBN) to remove the overhang and to generate a blunt end,  
 and cut with *Eco*RI. The resulting 849 bp fragment was  
 purified. The SLT-containing fragment from pING4407 was  
 excised by cutting with *Eag*I, blunted with T4 polymerase,  
 30 cut with *Xho*I, and the approximately 850 bp fragment which  
 resulted was purified. The two fragments were ligated  
 together into pING3197, which had been treated with *Eco*RI  
 and *Xho*I to generate pING4642. The DNA sequence at the  
*Bsp*HI-T4-MBN/*Eag*I junction revealed that two of the  
 35 expected codons were missing but that the fusion protein  
 was in frame.

-108-

C. Construction of  
SCA(V<sub>B</sub>-V<sub>L</sub>)::SLT::Gelonin Vectors

The expression vector containing the SCA(V<sub>B</sub>-V<sub>L</sub>)::SLT::Gelonin fusions was assembled using DNA from plasmids pING4636, (the *E. coli* expression vector for SCA(V<sub>B</sub>-V<sub>L</sub>)) and pING4407. Plasmid pING4636 was cut with *Bst*EII and *Xho*I and the resulting vector fragment was purified. Concurrently, pING4636 was used as a template for PCR with primers SCFV-7, 5'TGATGCGGCCGACATCTCAAGCTTGGTGC (SEQ ID NO: 112) and H65-G13, TGATGCGGCCGACATCTCAAGCTTGGTGC3' (SEQ ID NO: 113). The amplified product was digested with *Eag*I and *Bst*EII and the resulting approximately 380 bp fragment was purified. Plasmid pING4407 was then cut with *Eag*I and *Xho*I, resulting in an approximately 850 bp fragment, which was purified. The three above fragments were ligated together to produce pING4643.

D. Construction of  
SCA(V<sub>L</sub>-V<sub>B</sub>)::RMA::Gelonin Vectors

Expression vectors containing SCA(V<sub>L</sub>-V<sub>B</sub>)::RMA::Gelonin fusions were assembled using DNA from pING4640, pING4408 [Example 14A(iii)], and pING3825 (Example 2C). Plasmid pING4640 was cut with *Sal*I and *Bst*EII and the resulting approximately 700 bp vector fragment (containing the tetracycline resistance matter) was purified. Next, pING3825 was digested with *Nco*I and *Sal*I, resulting in an approximately 1344 bp fragment containing the 3' end of the gelonin gene and adjacent vector sequences. That fragment was purified. Plasmid pING4408 was then PCR amplified with oligonucleotide primers, RMA-G3 5'TCTAGGTCACCGTCTCCTCACCATCTGGACAGGCTGGA3' (SEQ ID NO: 114), and gelo-10. The resulting PCR product was cut with *Bst*EII and *Nco*I to generate an approximately 180 bp fragment containing the 3' end of V<sub>B</sub>, RMA, and the 5' end of the Gelonin gene which was purified. The above

-109-

three fragments were ligated to generate the final expression vector, pING4644.

E. Construction of  
SCA(V<sub>B</sub>-V<sub>L</sub>)::RMA::Gelonin Vectors

5 Expression vectors containing SCA(V<sub>B</sub>-  
V<sub>L</sub>)::RMA::Gelonin were constructed using DNA from pING 4636,  
pING4410, and pING3825. Plasmid pING4636 was digested with  
SalI and HindIII and the resulting vector fragment was  
10 purified. Next, pING3825 was cut with NcoI and SalI and  
the 1344 bp fragment which resulted contained the 3' end of  
the gelonin gene and adjacent vector sequences encoding  
tetracycline resistance was purified. Finally, pING4410  
was PCR amplified with primers RMA-G4,  
15 5'TTCGAAGCTTGAGATGAAACCATCTGGACAGGCTGGA3' (SEQ ID NO: 115)  
and gelo-10. The PCR product was cut with HindIII and  
NcoI, resulting in a 180 bp fragment containing the 3' end  
of V<sub>L</sub>, RMA, and the 5' end of Gelonin and was purified. The  
three above fragments were ligated together to generate the  
final expression vector, pING4645.

20 Gelonin::SCA fusions without a cleavable linker  
may be constructed by deletion of the SLT linker in  
pING4412 using the restriction enzymes EagI and FspI.  
Digestion at these sites and religation of the plasmid  
results in an in-frame deletion of the SLT sequence.

25 Example 17

Multivalent Immunofusions

Multivalent forms of the immunofusions may be  
constructed.

30 A. Construction of (Gel::RMA::kappa, Fd')<sub>2</sub>  
and (Gel::RMA::Fd', kappa), Expression Vectors

Bacterial Fab expressionn vectors can result in  
the production of F(ab')<sub>2</sub> if the two cysteine residues from  
the human IgG1 hinge region are included into the carboxyl-  
terminus of the Fd protein [Better et al., Proc. Natl.  
35 Acad. Sci. USA, 90:457-461 (1993)]. To express a gelonin

-110-

fusion protein that could form a bi-valent structure like an  $F(ab')_2$ , the he3 Fd' (2C) hinge region (Better et al., supra) was cloned into the expression vector pING3764 (Example 15C) encoding the fusion protein Gel::RMA::kappa, Fd.

Plasmid pING3764 was cut with XhoI and Bsu36I and the approximately 7500 bp fragment containing the immunofusion gene and vector sequences was purified. Plasmid pING4629, which encodes he3  $F(ab')_2$ , was also cut with Bsu36I and XhoI, and the approximately 200 bp DNA fragment containing the he3 Fd' (2C) gene segment was purified. These two DNA fragments were ligated to generate pING3775 encoding (Gel::RMA::kappa, Fd')<sub>2</sub>. An expression vector encoding the fusion protein (Gel::RMA::Fd', kappa)<sub>2</sub> was also made.

**B. Construction of Vectors Containing Both Gel::RMA::Fd and Gel::RMA::K Fusions**

In order to construct a plasmid comprising Gel::RMA::Fd and Gel::RMA::k fusions, plasmid pING3764 [described above in Example 15(b)] was digested with BsgI and SauI and a 5.7 kb vector fragment containing plasmid replication functions, Gel::RMA::k, and the 3' end of Fd was isolated and purified. Plasmid pING3768 [described above in Example 15(E)] was digested with SauI and partially digested with PstI and a 1.5 kb fragment containing Gel::RMA::Fd was purified. Finally, pING4000 [described above in Example 14] was digested with BsgI and PstI, generating a 350 bp fragment containing the 3' end of the kappa gene. That fragment was purified and the 5.7 kb, 1.5 kb, and 350 kb fragments described above were ligated together to form pING3770, containing the gelonin::RMA::k and gelonin:RMA::Fd fusions.

-111-

C. Construction of Vectors Containing Both Gel::SLT::Fd  
and Gel::SLT::k Fusions

Plasmid pING3772 contains the above-entitled fusions and was constructed as follows. Plasmid pING3763 [described above in Example 15(D)] was digested with BsgI and SauI and a 5.7 kb fragment containing the replication functions, the 5' end of Gel::SLT::k and the 3' end of Fd was generated and purified. Next, plasmid pING3767 [described in Example 15(D) above] was digested with SauI and PstI, generating a 1.5 kb fragment containing the 5' end of the gel::SLT::Fd fusion. That fragment was purified and pING4000 [described in Example 14 above] was digested with BsgI and PstI. The resulting 350 bp fragment was purified and the above-described 5.7 kb, 1.5 kb, and 350 bp fragments were ligated to form pING3772.

D. Expression of Multivalent Fusions

Both pING3770 and pING3772 were transformed into *E. coli* (E104) cells by techniques known to those of ordinary skill in the art and induced with arabinose. Concentrated supernatants from the transformed cell cultures were analyzed by Western blot analysis with rabbit anti-gelonin antiserum. Transformants from both plasmids generated a reactive band on the gel at the size expected for a Fab molecule carrying two gelonins (approximately 105 kD). These results are consistent with the production of fusion proteins comprising monovalent Fab, with both Fd and kappa chains separately fused to gelonin.

*E. coli* strains containing plasmids pING3775, pING3770 and pING3772 were grown in fermenters and the fusion protein products were purified. The (Gel::RMA::kappa,Fd')<sub>2</sub> expressed from pING3775 was purified as described in Better et al., *supra*.

-112-

Example 18Construction of Expression VectorsEncoding Immunofusions Without Linkers

5 Expression vectors encoding direct fusions of gelonin and dicistronic he3 Fab protein or single chain antibody were constructed as follows.

A.  $V_B V_L::Gel$ 

Plasmid pING4642 (Example 16B) which encodes the  $V_L V_B::SLT::Gel$  fusion protein was cut with *FspI* and *NcoI*,  
10 and the approximately 100 bp DNA fragment containing the 5'-end of the gelonin gene was purified. Plasmid pING4643 (Example 16C), which encodes the  $V_B V_L::SLT::Gel$  fusion protein, was cut with *EagI*, treated with T4 polymerase and cut with *PstI*. The approximately 850 bp DNA fragment  
15 encoding the  $V_B V_L$  gene segment was purified. The DNA fragments from pING4642 and pING4643 were ligated into the vector DNA fragment from pING4644 (Example 16D) [THIS PLASMID CONTRIBUTES THE REST OF THE GELONIN GENE THEN?] that had been cut with *PstI* and *NcoI* to generate pING3781,  
20 which encodes the  $V_B V_L::Gel$  direct gene fusion.

B.  $V_L V_B::Gel$ 

Plasmid pING4640 which encodes the he3 SCA gene  $V_L V_B$  was cut with *BspHI*, treated with T4 polymerase in the presence of the nucleotide dCTP only, treated with mung  
25 bean nuclease to remove the remaining 5' overhang, and then cut with *EcoRI*. The approximately 800 bp DNA fragment containing the he3  $V_L V_B$  gene was then purified on an agarose gel.

Plasmid pING3781 which encodes the direct fusion  $V_B V_L::Gel$  was digested with *EagI*, treated with T4  
30 polymerase, and then digested with *XhoI*. The approximately 800 bp DNA fragment encoding the gelonin gene was then purified on an agarose gel. The two DNA fragments from pING4640 and pING3781 were ligated into the vector DNA from pING3767 which had been digested *EcoRI* and *XhoI* and  
35 purified on an agarose gel. The resultant plasmid,

-113-

pING3348, encoded the  $V_L V_B::\text{Gel}$  fusion protein. The DNA sequence at the fusion junction was verified by direct DNA sequencing.

C.  $\text{Gel}::V_B V_L$

5           The plasmid pING3755 [Example 14B(iii)], which contains the gelonin gene with an engineered *EagI* site at its 3'-end, was cut with *EagI*, treated with T4 polymerase, and digested with *NcoI*. The approximately 650 bp DNA  
10 fragment containing the 3'-end of the gelonin gene was purified on an agarose gel. The plasmid pING4639 (Example 16A) encoding the fusion  $\text{Gel}::\text{SLT}::V_B V_L$  was cut with *XhoI* and then partially digested with *FspI*. The approximately 730 bp DNA fragment containing all of the he3  $V_B V_L$  gene was then purified in an agarose gel (a single *FspI* restriction  
15 site occurs in the  $V_B$  gene segment, and the purified he3  $V_B V_L$  gene was separated from the incomplete gene segment which was approximately 660 bp). The two DNA fragments from pING3755 and pING4639 were ligated into the vector pING3825 that had been digested with *NcoI* plus *XhoI* and purified on  
20 an agarose gel. The plasmid pING3350 was generated which encoded the  $\text{Gel}::V_B V_L$  fusion protein. The DNA sequence at the fusion junction was verified by direct DNA sequencing.

D.  $\text{Gel}::V_L V_B$

25           Plasmid pING3336 which encodes the he3  $V_L V_B$  single chain antibody gene was cut with *SstI* and *AseI*, and the approximately 5500 bp DNA fragment containing the 3'-end of  $V_L V_B$  and downstream vector sequences was purified. (pING3336 is identical to pING4640 except that the  $V_L V_B$  gene encodes six histidine residues in frame at the carboxyl-  
30 terminus). Plasmid pING4627 (Example 15A) served as a substrate for PCR amplification of the  $V_B$  gene segment. Plasmid pING4627 was amplified with the two oligonucleotide primers HUK-7 (SEQ ID NO: 92) and JK1-HindIII (SEQ ID NO: 87), the resultant product was treated with T4 polymerase  
35 and cut with *AseI*, and the 86 bp DNA fragment containing

-114-

the 5'-end of the  $V_L$  was purified. The DNA fragments from pING3336 and pING4627 were ligated to the approximately 2350 bp DNA fragment of pING3755 generated by digestion with *EagI*, treatment with T4 polymerase and subsequent digestion with *SstI*. The resultant vector containing the Gel:: $V_LV_B$  gene fusion was named pING4652. The DNA sequence of pING4652 was verified at ligation junctions.

E. Gel:: $\kappa$ , Fd

The direct gene fusion which encodes Gel:: $\kappa$ , Fd was also assembled from DNA segments from three plasmids. Plasmid pING3764 (Example 15C) was digested with *HindIII* and *XhoI*, and the approximately 1200 bp DNA fragment encoding the 3'-end of the  $\kappa$  gene and the Fd gene was purified. Plasmid pING4652, which encodes a direct gene fusion of gelonin to the he3 SCA gene  $V_LV_B$ , was cut with *BglIII* and *HindIII*, and the approximately 850 bp DNA fragment encoding the 3'-end of the gelonin gene and the  $V_L$  region of  $\kappa$  was purified. The DNA fragments from pING3764 and pING4652 were ligated into the vector fragment from pING3825 (Example 2C) that had been digested with *BglIII* and *XhoI* to generate pING3784 encoding Gel:: $\kappa$ , Fd.

F. Gel:: $\kappa$ , Fd

Plasmid pING3768 (Example 15F), which encodes the fusion protein Gel:: $\kappa$ , Fd, was cut with *NdeI* and *NheI*, and the DNA segment containing the majority of the he3 Fd gene, the he3  $\kappa$  gene and a portion of the tetracycline resistance gene of the vector was purified. Plasmid pING3350, which is described in section C above, was cut with *NdeI* and *PstI*, and the DNA fragment containing the 5'-end of the he3 Fd gene linked to the gelonin gene was purified. The DNA fragments from pING3350 and pING3768 were ligated into the vector fragment from pING4633 (Example 16D) that had been cut with *NheI* and *PstI* to



-115-

generate pING3789. Plasmid pING3789 encodes the fusion protein Gel::Fd, kappa.

---

#### Example 19

##### Alternative Cathepsin Cleavable Linkers

5           The segment of rabbit muscle aldolase chosen for the RMA linker described herein is known to contain peptide sequences susceptible to digestion with cathepsins. Other cathepsin-cleavable protein segments are effective targets for intracellular cleavage, and two particular amino acid  
10 sequences were included as cleavable linkers in additional immunofusions of the invention. These are the amino acid sequence KPAKFFRL (SEQ ID NO: 141 ("CCF") and KPAKFLRL (SEQ ID NO: 142) ("CCL"). Two oligonucleotides were synthesized that encode these peptide segments. Degeneracy was  
15 introduced at one nucleotide position in each synthetic primer to allow the appropriate amino acid to be encoded at the particular amino acid position in which CCF and CCL differ. The two oligonucleotides 5'-GGCCGCAAAGCCGGCTAAGTTCTT(A/C)CGTCTGAGT-3' (SEQ ID NO: 143)  
20 and 5'-ACTCAGACG(G/T)AAGAACTTAGCCGGCTTTGC-3' (SEQ ID NO: 144). The oligonucleotide linkers were then used to assemble a family of fusion gene expression vectors encoding: Gel::CCL::kappa, Fd; Gel::CCF::kappa, Fd; Gel::CCF::V<sub>L</sub>V<sub>H</sub>; and Gel::CCL::V<sub>H</sub>V<sub>L</sub>.

25           The CCL and CCF linkers were also included in fusion vectors where the antigen-binding domain of the fusion protein was at the N-terminus of the fusion to generate expression vectors encoding immunofusions such as V<sub>L</sub>V<sub>H</sub>::CCL::Gel.

30           Several of the fusion proteins with the CCL and CCF linkers were tested for cytotoxicity on the T cell lines HSB2 and PBMC and were comparable in activity to the fusion proteins containing the RMA linker.

-116-

Example 20Expression And Purification Of Gelonin ImmunofusionsA. Expression Of Gelonin Immunofusions

5 Each of the gelonin gene fusions whose construction is described in Example 15 was co-expressed with its pair H65 Fab gene in arabinose-induced *E. coli* strain E104.

10 Expression products of the gene fusions were detected in the supernatant of induced cultures by ELISA. Typically, a plate was coated with antibody recognizing gelonin. Culture supernatant was applied and bound Fab was detected with antibody recognizing human kappa coupled to horseradish peroxidase. H65 Fab fragment chemically conjugated to gelonin was used a standard. Alternative  
15 ELISA protocols involving coating a plate with antibody recognizing either the kappa or Fd or involving a detection step with anti-human Fd rather than anti-human kappa yielded similar results. Only properly assembled fusion protein containing gelonin, kappa and Fd was detected by  
20 this assay. Unassociated chains were not detected.

The fusion protein produced from induced cultures containing expression vectors pING4406, 4407, 4408, and 4410 in *E. coli* E104 accumulated at about 20-50 ng/ml. The fusion proteins expressed upon induction of pING3754, 3334,  
25 3758 and 3759 (but not pING3757) were expressed at much higher levels, at about 100 to 500 ng/ml. A fusion protein of about 70,000 Kd was detected in the concentrated *E. coli* culture supernatant by immunostaining of Western blots with either anti-human kappa or anti-gelonin antibodies.

30 The Gelonin::SLT::Fd' (kappa) fusion protein from pING3754 (ATCC 69102) was purified from induced 10 L fermentation broth. The 10 L fermentation broth was concentrated and buffer exchanged into 10mM phosphate buffer at pH 7.0, using an S10Y10 cartridge (Amicon) and a  
35 DC10 concentrator. The supernatant was purified by passing the concentrated supernatant through a DE52 column (20 x 5 cm) equilibrated with 10 mM sodium phosphate buffer at pH

-117-

7.0. The flow-through was then further purified and concentrated by column chromatography on CM52 (5 x 10 cm) in 10 mM phosphate buffer. A 0 - 0.2 M linear gradient of NaCl was used to elute the fusion protein, and fractions containing the fusion protein were pooled and loaded onto a Protein G column (1ml). The fusion protein was eluted from protein G with 0.2 M sodium citrate, pH 5.5 and then 0.2 M sodium acetate, pH 4.5, and finally, 0.2 M glycine, pH 2.5. The Gelonin::RMA::Fd' (kappa) and Gelonin::RMA::kappa (Fd') fusions proteins were purified from fermentation broths by similar methods except that the CM52 column step was eliminated, and the DE52 column was equilibrated with 100mM sodium phosphate buffer at pH 7.0. The fusion proteins were not purified to homogeneity.

Each of the three purified fusion proteins was then assayed for activity in the RLA assay and for cytotoxicity against the T-cell line HSB2. (T cells express the CD5 antigen which is recognized by H65 antibody.) The RLA assay was performed as described in Example 4 and results of the assay are presented below in Table 12.

Table 12

	<u>Fusion Protein</u>	<u>IC50 (pM)</u>
	rGelonin	11
25	Gelonin::SLT::Fd (kappa)	19
	Gelonin::RMA::Fd (kappa)	28
	Gelonin::RMA::kappa (Fd)	10

Two fusion proteins were tested in whole cell cytotoxicity assays performed as described in Example 6 (Table 13). As shown in Table 13, the fusion proteins were active. Gelonin::SLT::Fd(kappa) killed two T cell lines, HSB2 and CEM, with respective IC<sub>50</sub>s 2-fold (HSB2) or 10-fold (CEM) higher than that of the gelonin chemically linked to H65. See Table 13 below for results wherein IC<sub>50</sub> values were

-118-

adjusted relative to the amount of fusion protein in each sample.

Table 13IC<sub>50</sub> (pMT)

5	<u>Fusion Protein</u>	<u>HSB2 Cells</u>	<u>CEM Cells</u>
	he3Fab-Gel <sub>A50(C44)</sub>	165	173
	Gelonin::SLT::Fd (kappa)	180	1007
	Gelonin::RMA::Fd (kappa)	150	NT

These fusion protein showed similar activity on peripheral blood mononuclear cells (data not shown).

#### B. Purification of Immunofusions

##### (i) Immunofusions Comprising CH65Fab'

Immunofusions comprising a CH65Fab' fragment were purified from cell-free supernatants by passing the supernatant through a CM Sphradex (Sepacor) column (5cm x 3cm), equilibrated in 10 mM Na phosphate at pH 7.0. Immunofusion proteins bind to the column and are eluted with 10 mM Na phosphate, 200 mM NaCl, pH 7.0. The eluate was diluted two-fold with 20 mM HEPES, 3 M ammonium sulfate, pH 7.6 and loaded onto a phenyl sepharose fast flow (Pharmacia) column (2.5 x3.5 cm), equilibrated in 20 mM HEPES, 1.2 M ammonium sulfate, pH 7.0. The column was next washed with 20 mM Hepes, 1.2 M ammonium sulfate, pH 7.0 and eluted with 20 mM HEPES, 0.9 M ammonium sulfate, pH 7.0. The phenyl sepharose eluate was concentrated to a volume of 2-4 ml in an Amicon stirred cell fitted with a YM10 membrane. The concentrated sample was loaded onto an S-200 (Pharmacia) column (3.2x 38 cm), equilibrated in 10 mM Na phosphate, 150 mM NaCl, pH 7.0. The column was run in the same buffer and fractions were collected. Fractions containing the fusion protein of desired molecular weight were combined. For example, by selection of appropriate column fractions, both monovalent (gelonin-Fab') and

-119-

bivalent (gelonin<sub>2</sub>-F(ab')<sub>2</sub>) forms encoded by pING3758 were purified.

(ii) Immunofusions Comprising he3Fab

Immunofusions comprising he3Fab were purified as  
5 in the preceding section with the exception that the phenyl  
sepharose column was eluted with 20 mM HEPES, 1.0 M  
ammonium sulfate, pH 7.0.

(iii) Immunofusions Comprising SCA

Cell-free supernatant was passed through a CM  
10 spheradex column (5 x 3 cm), equilibrated with 10 mM Na  
phosphate, pH 7.0. Single-chain antibody binds to the  
column which is then washed with 10 mM Naphosphate, 45 mM  
NaCl, pH 7.0. The fusion protein was then eluted with 10  
mM Naphosphate, 200 mM NaCl, pH 7.0. The eluate was  
15 diluted two-fold with 20 mM HePES, 3 M ammonium sulfate, pH  
7.0 and loaded onto a butyl sepharose Fast Flow (Pharmacia)  
column (2.5 x 4.1 cm) equilibrated in 20 mM HEPES, 1.5 M  
ammonium sulfate, pH 7.0. The column was then washed with  
20 mM HEPES, 1.0 M ammonium sulfate, pH 7.0 and eluted with  
20 20 mM HEPES pH 7.0. The butyl sepharose eluate was  
concentrated to a volume of 2-4 ml in an Amicon stirred  
cell fitted with a YM10 membrane. The concentrated sample  
was loaded onto an S-200 (Pharmacia) column (3.2 x 38 cm)  
equilibrated in 10 mM Na phosphate, 150 mM NaCl, pH 7.0.  
25 The column was then run in the same buffer and the  
fractions were collected. Some of the fractions were  
analyzed by SDS-PAGE to determine which fractions to pool  
together for the final product.

Example 21

30 Activity of Gelonin Immunofusions

A concern in constructing immunofusions  
comprising any RIP is that the targeting and enzymatic  
activities of the components of the fusion protein may be  
lost as a result of the fusion. For example, attachment of

-120-

an RIP to the amino terminus of an antibody may affect the antigen-binding (complementarity-determining regions) of the antibody and may also result in steric hinderance at the active site. Similarly, the activity of an RIP may be hindered by attachment of an antibody or antibody portion. For example, RIPs chemically conjugated to antibodies via a disulfide bridge are typically inactive in the absence of reducing agents. In order to assess the foregoing immunofusions of the present invention, such proteins were subjected to assays to determine their enzymatic, binding, and cytotoxic activities.

#### A. Reticulocyte Lysate Assay

The enzymatic activity of immunofusions comprising gelonin was assayed using the reticulocyte lysate assay (RLA) describe above. As noted in Example 4, the RLA assay measures the inhibition of protein synthesis in a cell-free system using endogenous globin mRNA from a rabbit red blood cell lysate. Decreased incorporation of tritiated leucine ( $^3\text{H}$ -Leu) was measured as a function of toxin concentration. Serial log dilutions of standard toxin (the 30 kD form of ricin A-chain, abbreviated as RTA 30), native gelonin, recombinant gelonin (rGelonin or rGel) and gelonin analogs were tested over a range of 1  $\mu\text{g/ml}$  to 1 pg/ml. Samples were tested in triplicate, prepared on ice, incubated for 30 minutes at 37°C, and then counted on an Inotec Trace 96 cascade ionization counter. By comparison with an uninhibited sample, the picomolar concentration of toxin (pM) which corresponds to 50% inhibition of protein synthesis ( $\text{IC}_{50}$ ) was calculated.

Representative data for various immunotoxins of the invention are shown below in Table 14 .

-121-

Table 14

	<u>Immunotoxin</u>	<u>Lot No.</u>	<u>IC<sub>50</sub>(pM)</u>
	rGel::RMA::SCA(V <sub>B</sub> -V <sub>L</sub> )	AB1136	12
	rGel::RMA::SCA(V <sub>L</sub> -V <sub>B</sub> )	AB1137	18
5	rGel::SLT::SCA(V <sub>B</sub> -V <sub>L</sub> )	AB1133	26
	rGel::RMA::SCA(V <sub>L</sub> -V <sub>B</sub> )	AB1124	33
	rGel::RMA::K+Fd' (cH65Fab')	AB1122	54
	rGel::SLT::K+Fd(he3Fab)	AB1160	40
	rGel::RMA::K+Fd(he3Fab)	AB1149	33
10	rGel::RMA::Fd+K(he3Fab)	AB1163	14
	rGel::Fd'+K(cH65Fab')	AB1123	45

Contrary to the expectations discussed above, gelonin immunofusions of the invention exhibit enzymatic activity which is comparable to the activities of native and recombinant gelonin shown in Example 4. This was true for fusions made with either the reducible (SLT) or non-reducible (RMA) linkers.

#### B. Binding Activity of Immunofusions

Several immunofusions according to the present invention were assayed for their ability to compete with labelled antibody for binding to CD5-positive cells. The K<sub>d</sub> of the immunofusions was estimated by three different means as shown in Table 15. The first K<sub>d</sub> estimation (K<sub>d1</sub> in Table 15) was obtained by competition with fluorescein-labelled H65 IgG for binding to MOLT-4X cells (ATCC CRL 1582) according to the procedure reported in Knebel, et al., *Cytometry Suppl.*, 1: 68 (1987), incorporated by reference herein.

The second K<sub>d</sub> measurement (K<sub>d2</sub> in Table 15) was obtained by Scatchard analysis of competition of the immunofusion with <sup>125</sup>I-CH65 IgG for binding on MOLT-4M cells as follows. A 20 µg aliquot of chimeric H65 IgG (CH65 IgG) was iodinated by exposure to 100 µl lactoperoxidase-glucose oxidase immobilized beads (Enzymobeads, BioRad), 100 µl of PBS, 1.0 mCi I<sup>125</sup> (Amersham, IMS30), 50 µl of 55 mM

-122-

b-D-glucose for 45 minutes at 23°C. The reaction was quenched by the addition of 20  $\mu$ l of 105 mM sodium metabisulfite and 120 mM potassium iodine followed by centrifugation for 1 minute to pellet the beads.  $^{125}$ I-CH65 IgG was purified by gel filtration using a 7 ml column of sephadex G25, eluted with PBS (137 mM NaCl, 1.47 mM  $\text{KH}_2\text{PO}_4$ , 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 2.68 mM KCl at pH 7.2-7.4) plus 0.1% BSA.  $^{125}$ I-CH65 IgG recovery and specific activity were determined by TCA precipitation.

Competitive binding was performed as follows: 100  $\mu$ l of Molt-4M cells were washed two times in ice-cold DHB binding buffer (Dubellco's modified Eagle's medium (Gibco, 320-1965PJ), 1.0% BSA and 10 mM Hepes at pH 7.2-7.4). Cells were resuspended in the same buffer, plated into 96 v-bottomed wells (Costar) at  $3 \times 10^5$  cells per well and pelleted at 4°C by centrifugation for 5 min at 1,000 rpm using a Beckman JS 4.2 rotor; 50  $\mu$ l of 2X-concentrated 0.1 nM  $^{125}$ I-CH65 IgG in DHB was then added to each well and competed with 50  $\mu$ l of 2X - concentrated CH65 IgG in DHB at final protein concentrations from 100 nM to 0.0017 nM. The concentrations of assayed proteins were determined by measuring absorbance ( $A_{280}$ ) and using an extinction coefficient of 1.0 for fusion proteins, 1.3 for Fab, and 1.22 for Fab conjugated to gelonin. Also, protein concentrations were determined by BCA assay (Pierce Chemical) with bovine serum albumin as the standard. Binding was allowed to proceed at 4°C for 5 hrs and was terminated by washing cells three times with 200  $\mu$ l of DHB binding buffer by centrifugation for 5 min. at 1,000 rpm. All buffers and operations were at 4°C. Radioactivity was determined by solubilizing cells in 100  $\mu$ l of 1.0 M NaOH and counting in a Cobra II auto gamma counter (Packard). Data from binding experiments were analyzed by the weighted nonlinear least squares curve fitting program, MacLigand, a Macintosh version of the computer program "Ligand" from Munson, *Analyt. Biochem.*, 107:220 (1980), incorporated by reference herein.



-123-

Finally, the  $K_d$  ( $K_{d3}$  in the Table) was estimated by examination of the  $ED_{50}$  values obtained from separate competition binding assays, performed as described in the previous paragraph. All three measurements are shown in Table 15 below:

Table 15

	<u>Molecule Type</u>	<u><math>K_{d1}</math></u>	<u><math>K_{d2}</math></u>	<u><math>K_{d3}</math></u>
	H65 IgG	1.6	ND	ND
	CH65 IgG	ND	3.0	2.5
10	CH65Fab'	4.0	14.0	ND
	CH65Fab'-rGel <sub>A50(C44)</sub>	3.5	13.0	ND
	rGel::RMA::K+Fd' (CH65Fab')	16.0	ND	100
	he3Fab	1.20	2.60	ND
	he3Fab-rGel <sub>A50(C44)</sub>	1.10	2.70	ND
15	rGel::RMA::K+Fd' (he3Fab)	2.60	ND	5.0
	rGel::SLT::K+Fd (he3Fab)	ND	ND	30
	SCA( $V_L-V_B$ )	2.20	ND	30
	rGel::RMA::SCA( $V_B-V_L$ )	3.50	ND	20
	rGel::RMA::SCA( $V_L-V_B$ )	4.70	ND	30
20	SCA( $V_L-V_B$ )	ND	ND	20
	rGel::RMA::SCA( $V_L-V_B$ )	2.30	ND	ND
	ND = not determined			

The results presented in Table 15 suggest that Fab and SCA antibody forms may retain substantial binding activity even when fused to an RIP.

### C. Comparative Cytotoxicity Assays

Fusion proteins and immunoconjugates according to the present invention were used in a comparative cytotoxicity assay. Two types of assays were conducted, one targeting T cell line HSB2, and the other targeting lectin-activated peripheral blood mononuclear cells (PBMC) according to procedures in Example 6. The results of the assays are presented below in Tables 16a, 16b and 16c.

Table 16a

**CYTOTOXIC POTENCIES:  
CHEMICAL VS. GENE-FUSED CONJUGATES**

Immunotoxin	Lot #	HSB2			
		Fusion Plasmid	IC <sub>50</sub> , pM Toxin	IC <sub>50</sub> , pM Toxin	N
CD5 Plus	HF002002	---	148	24	8
H65-M-rGel <sub>A50(C44)</sub>	999	---	68*	NA	1
CH65-MM-rGel	807	---	183*	NA	1
CH65Fab'-M-rGel <sub>A50(C44)</sub>	941	---	99	6	2
He2Fab-M-rGel <sub>A50(C44)</sub>	970	---	468	195	4
he3Fab-M-rGel <sub>A50(C44)</sub>	1012/1047	---	190	70	12
he3Fab-SMCC-rGel <sub>A50(C44)</sub>	1086	---	5,904	2,442	2
rGel::SLT::Fd'+K(1) <sup>+</sup>	AB1095	PING3754	320	25	2
rGel::SLT::Fd'+K(3) <sup>+</sup>	AB1095	PING3754	374*	NA	1
rGel::SLT::K+Fd(he3)	AB1147	PING3763	495*	NA	1
rGel::SLT::K+Fd(he3)	AB1160	PING3763	746*	NA	1
rGel::SLT::SCA(Vh-VL)	AB1133	PING4639	422	31	5
rGel::SLT::SCA(VL-Vh)	AB1124	PING4412	776	347	3
rGel::RMA::K+Fd'	AB1122	PING3758	1,506	1,033	2
rGel::RMA::K+Fd'	AB1141	PING3758	5,833*	NA	1
rGel::RMA::K+Fd(he3)	AB1149	PING3764	9,154*	NA	1

		PBM <sub>C</sub>			
Immunotoxin	Lot #	IC <sub>50</sub> , pM Toxin	IC <sub>50</sub> , pM Toxin	SD	N
rGel::RMA::K+Fd(he3)	AB1149	7,124	10,764	14,081	5
rGel::RMA::K+Fd(he3)	AB1161	1,854	2,990	3,324	3
rGel::RMA::Fd'+K	RF524(1)	1,760	1,893	1,049	5
rGel::RMA::Fd'+K	AB1121	2,090	1,664	1,553	6
rGel::RMA::Fd+K(he3)	AB1163	854	567	406	2
rGel::RMA::SCA(Vh-Vl)	AB1136	393	567	510	7
rGel::RMA::SCA(Vh-Vl)	AB1152	9,650	9,170	6,483	3
rGel::RMA::SCA(Vl-Vh)	AB1137	4,040	4,554	4,310	7
rGel::RMA::SCA(Vl-Vh)	AB1164	1,598	1,598	1,144	2
rGel::Fd'+K	AB1123	2,606	2,777	2,167	4
K::RMA::rGel+Fd	AB1140	1,545	1,545	417	2
rGel	1056	13,350	40,233	43,048	6
8B2.3Fab-m-rGel <sub>A50(C44)</sub>	1057	12,400	13,174	14,339	11

\* -- Results represent single values and not a mean value.  
 \* -- rGel::SLT::Fd'+k(1) and rGel::SLT::Fd'+k(3) are separate fractions from the final purification column.

Table 16c

Fusion Plasmid	Immunotoxin	HSB2 IC50, pM Toxin	n=	PBMC IC50, pM Toxin	n=
PING4644	V <sub>L</sub> V <sub>H</sub> ::RMA::Gel	1933	4	1513	29
PING3784	Gel::kappa, Fd	>12,500	3	2645	7
PING3789	Gel::Fd, kappa	1212	1	3665	1
PING3348	V <sub>L</sub> V <sub>H</sub> ::Gel	2158	4	1264	9
PING3350	Gel::V <sub>H</sub> V <sub>L</sub>	8056	3	2729	4
PING3775	(Gel::RMA::kappa, Fd') <sub>2</sub>	175	1	44	22
PING3770	Gel::RMA::k, Gel::RMA::Fd	3548	2	519	9
PING3772	Gel::SLT::k, Gel::SLT::Fd	-	-	663	6

-129-

The results presented in Tables 16a, 16b and 16c demonstrate that gelonin immunofusions may vary in their activity. In general, immunofusions of the invention which have IC<sub>50</sub> median or mean values of less than 2000 pM Toxin display strong activity; whereas those with IC<sub>50</sub> values equal to or less than 500 pM Toxin are considered highly active. In sum, the results in Tables 16a, 16b and 16c demonstrate that the optimum fusion protein for killing a particular cell line may vary depending upon the targeted cell.

### Example 22

#### Preparation Of BRIP

BRIP possesses characteristics which make it an attractive candidate for a component of immunotoxins. BRIP is a naturally unglycosylated protein that may have reduced uptake in the liver and enhanced circulatory residence time *in vivo*. Additionally, BRIP is less toxic and less immunogenic in animals than the A-chain of ricin. Cloning of the BRIP gene and expression of recombinant BRIP in an *E. coli* expression system obviates the need to purify native BRIP directly from barley, and enables the development of analogs of BRIP which may be conjugated with an available cysteine residue for conjugation to antibodies.

#### A. Purification Of BRIP And Generation Of Polyclonal Antibodies To BRIP

Native BRIP was purified from pearled barley flour. Four kilograms of flour was extracted with 16 liters of extraction buffer (10 mM NaPO<sub>4</sub>, 25 mM NaCl, pH 7.2) for 20 hours at 4°C. The sediment was removed by centrifugation, and 200 ml of packed S-Sepharose (Pharmacia, Piscataway, New Jersey) was added to absorb BRIP. After mixing for 20 hours at 4°C, the resin was allowed to settle out, rinsed several times with extraction buffer and then packed into a 2.6 x 40 cm column. Once packed, the column was washed with extraction buffer (150

-130-

ml/h) until the absorbance of the effluent approached zero. BRIP was then eluted with a linear gradient of 0.025 to 0.3 M NaCl in extraction buffer and 5 ml fractions were collected. BRIP-containing peaks (identified by Western analysis of column fractions) were pooled, concentrated to about 20 ml, and then chromatographed on a 2.6 x 100 cm Sephacryl S-200HR (Pharmacia) column equilibrated in 10 mM NaPO<sub>4</sub>, 125 mM NaCl, pH 7.4 (10 ml/hr). BRIP-containing peaks were pooled again, concentrated, and stored at -70°C.

The resulting purified BRIP protein had a molecular weight of about 30,000 Daltons, based upon the mobility of Coomassie-stained protein bands following SDS-PAGE. The amino acid composition was consistent with that published by Asano et al., *Carlsberg Res. Comm.*, 49:619-626 (1984).

Rabbits were immunized with purified BRIP to generate polyclonal antisera.

#### B. Cloning Of The BRIP Gene

A cDNA expression library prepared from germinating barley seeds in the phage  $\lambda$  expression vector  $\lambda$ ZAPII was purchased from Stratagene, La Jolla, CA. Approximately 700,000 phage plaques were screened with anti-BRIP polyclonal antisera and 6 immunoreactive plaques were identified. One plaque was chosen, and the cDNA contained therein was excised from  $\lambda$ ZAPII with EcoRI and subcloned into pUC18 generating the vector pBS1. The cDNA insert was sequenced with Sequenase (United States Biochemical, Cleveland, Ohio). The DNA sequence of the native BRIP gene is set out in SEQ ID NO: 12. To confirm that cDNA encoded the native BRIP gene, the cDNA was expressed in the *E. coli* plasmid pKK233-2 (Pharmacia). BRIP protein was detected in IPTG-induced cells transformed with the plasmid by Western analysis with above-described rabbit anti-BRIP antisera.

-131-

C. Construction Of An *E. coli* Expression Vector Containing The BRIP Gene

Barley cDNA containing the BRIP gene was linked to a *pelB* leader sequence and placed under control of an *araB* promoter in a bacterial secretion vector.

An intermediate vector containing the BRIP gene linked to the *pelB* leader sequence was generated. Plasmid pBS1 was cut with *NcoI*, treated with Mung Bean Nuclease, cut with *BamHI* and the 760 bp fragment corresponding to amino acids 1-256 of BRIP was purified from an agarose gel. Concurrently, a unique *XhoI* site was introduced downstream of the 3'-end of the BRIP gene in pBS1 by PCR amplification with a pUC18 vector primer (identical to the Reverse® primer sold by NEB or BRL but synthesized on a Cyclone Model 8400 DNA synthesizer) and the specific primer BRIP 3'*Xho*. The sequence of each of the primers is set out below.

Reverse (SEQ ID NO: 45)

5' AACAGCTATGACCATG 3'

BRIP 3'*Xho* (SEQ ID NO: 46)

5' TGAAGCTCGAGGAAACTACCTATTTCCCAC 3'

Primer BRIP 3'*Xho* includes a portion corresponding to the last 8 bp of the BRIP gene, the termination codon and several base pairs downstream of the BRIP gene, and an additional portion that introduces a *XhoI* site in the resulting PCR fragment. The PCR reaction product was digested with *BamHI* and *XhoI*, and an 87 bp fragment containing the 3'-end of the BRIP gene was purified on a 5% acrylamide gel. The 760 and 87 bp purified BRIP fragments were ligated in the vector pING1500 adjacent to the *pelB* leader sequence. pING1500 had previously been cut with *SstI*, treated with T4 polymerase, cut with *XhoI*, and purified. The DNA sequence at the junction of the *pelB* leader and the 5'-end of the BRIP gene was verified by DNA sequence analysis. This vector was denoted pING3321-1.

The final expression vector was assembled by placing the BRIP gene under the control of the inducible *araB* promoter. Plasmid pING3321-1 was cut with *PstI* and

-132-

XhoI, and the BRIP gene linked to the pelB leader was purified from an agarose gel. The expression vector pING3217, containing the araB promoter, was cut with PstI and XhoI and ligated to the BRIP gene. The expression  
5 vector was denoted pING3322.

Arabinose induction of *E. coli* cells containing the plasmid pING3322 in a fermenter resulted in the production of about 100 mg per liter of recombinant BRIP. *E. coli*-produced BRIP displays properties identical to BRIP  
10 purified directly from barley seeds.

#### D. Construction Of BRIP Analogs With A Free Cysteine Residue

The BRIP protein contains no cysteine residues, and therefore contains no residues directly available which  
15 may form a disulfide linkage to antibodies or other proteins. Analogs of recombinant BRIP were generated which contain a free cysteine residue near the C-terminus of the protein. Three residues of the BRIP protein were targets for amino acid substitutions. Comparison of the amino acid  
20 sequence of BRIP to the known tertiary structure of the ricin A-chain (see FIG. 2) suggested that the three positions would be available near the surface of the molecule. The three BRIP analogs include cysteines substituted in place of serine<sub>277</sub>, alanine<sub>270</sub>, and leucine<sub>256</sub>  
25 of the native protein, and were designated BRIP<sub>C277</sub> (SEQ ID NO: 127), BRIP<sub>C270</sub> (SEQ ID NO: 128) and BRIP<sub>C256</sub> (SEQ ID NO: 129), respectively.

(1) A plasmid vector capable of expressing the BRIP<sub>C277</sub> analog was constructed by replacing the 3'-end of  
30 the BRIP gene with a DNA segment conferring the amino acid change. The EcoRI fragment containing the BRIP gene from pBS1 was subcloned into M13mp18, and single-stranded DNA (anti-sense strand) was amplified by PCR with primers OBM2 (corresponding nucleotides -11 to +8 of the BRIP gene) and  
35 OMB4 (corresponding to amino acids 264-280 of BRIP and the termination codon of BRIP, and incorporating the



-133-

substitution of a cysteine codon for the native codon for serine<sub>277</sub> of native BRIP). The sequences of primers OBM2 and OMB4, wherein the underlined nucleotides encode the substituted cysteine, are set out below.

5 OBM2 (SEQ ID NO: 47)  
5' GCATTACATCCATGGCGGC 3'  
OMB4 (SEQ ID NO: 48)  
5' GATATCTCGAGTTAACTATTTCCACACACG  
CATGGAACAGCTCCAGCGCCTTGGCCACCGTC 3'

10 A fragment containing a BRIP gene in which the codon for the amino acid at position 277 was changed to a cysteine codon was amplified. The fragment was cloned into the *Sma*I site of pUC19 (BRL) and the plasmid generated was denoted pMB22. pMB22 was digested with *Eco*RI and an *Eco*RI-*Xho*I linker (Clonetech, Palo Alto, CA) was ligated into the  
15 vector. Subsequent digestion with *Xho*I and religation generated vector pINGMB2X. A *Bam*HI to *Xho*I fragment encoding the 3'-end of BRIP with the altered amino acid was excised from pMB2X and the fragment was purified on a 5%  
20 acrylamide gel. This fragment along with an *Eco*RI to *Bam*HI fragment containing the *pelB* leader sequence and sequences encoding the first 256 amino acids of BRIP were substituted in a three piece ligation into pING3322 cut with *Eco*RI and *Xho*I. The resulting vector containing the BRIP<sub>c277</sub> analog  
25 was designated pING3803 (ATCC Accession No. 68722).

(2) A BRIP analog with a free cysteine at position 256 was constructed using PCR to introduce the amino acid substitution. A portion of the expression plasmid pING3322 was amplified with primers BRIP-256 and  
30 *HIND*III-2. The sequence of each primer is set out below.

BRIP-256 (SEQ ID NO: 49)  
5' TGTCTGTTTCGTGGAGGTGCCG 3'  
*HIND*III-2 (SEQ ID NO: 44)  
5' CGTTAGCAATTTAACTGTGAT 3'

35 Nucleotides 4-21 of primer BRIP-256 encode amino acids 256-262 of BRIP while the underlined nucleotides specify the cysteine to be substituted for the leucine at the

-134-

corresponding position of the native BRIP protein. Primer *HINDIII*-2 corresponds to a portion of the plasmid. The PCR product, which encodes the carboxyl terminal portion of the BRIP analog, was treated with T4 polymerase, cut with *XhoI*, and the resulting fragment was purified on a 5% acrylamide gel. Concurrently, plasmid pING3322 was cut with *BamHI*, treated with T4 polymerase, cut with *EcoRI*, and the fragment containing the *pelB* leader sequence and sequences encoding the first 256 amino acids of BRIP was purified. The two fragments were then assembled back into pING3322 to generate the gene encoding the analog BRIP<sub>C256</sub>. This plasmid is denoted pING3801.

(3) A BRIP analog with a cysteine at position 270 was also generated using PCR. A portion of the expression plasmid pING3322 was amplified with primers BRIP-270 and the *HINDIII*-2 primer (SEQ ID NO: 44). The sequence of primer BRIP-270 is set out below.

BRIP-270 (SEQ ID NO: 50)

5' CCAAGTGTCTGGAGCTGTTCCATGCGA 3'

Primer BRIP-270 corresponds to amino acids 268-276 of BRIP with the exception of residue 270. The codon of the primer corresponding to position 270 specifies a cysteine instead of the alanine present in the corresponding position in native BRIP. The PCR product was treated with T4 polymerase, cut with *XhoI*, and the 51 bp fragment, which encodes the carboxyl terminal portion of the analog, was purified on a 5% acrylamide gel. The fragment (corresponding to amino acids 268-276 of BRIP<sub>C270</sub>) was cloned in a three piece ligation along with the internal 151 bp BRIP restriction fragment from *SstII* to *MscI* (corresponding to BRIP amino acids 217-267) from plasmid pING3322, and restriction fragment from *SstII* to *XhoI* from pING3322 containing the remainder of the BRIP gene. The plasmid generated contains the gene encoding the BRIP<sub>C270</sub> analog and is designated pING3802.

-135-

E. Purification Of Recombinant  
BRIP And The BRIP Analogs

~~Recombinant BRIP (rBRIP) and the BRIP analogs~~  
with free cysteine residues were purified essentially as  
described for native BRIP except they were prepared from  
concentrated fermentation broths. For rBRIP, concentrated  
broth from a 10 liter fermentation batch was exchanged into  
10 mM Tris, 20 mM NaCl pH 7.5, loaded onto a Sephacryl S-  
200 column, and eluted with a 20 to 500 mM NaCl linear  
gradient. Pooled rBRIP was further purified on a Blue  
Toyopearl® column (TosoHaas) loaded in 20 mM NaCl and  
eluted in a 20 to 500 mM NaCl gradient in 10mM Tris, pH  
7.5. For BRIP analogs, concentrated fermentation broths  
were loaded onto a CM52 column (Whatman) in 10 mM phosphate  
buffer, pH 7.5, and eluted with a 0 to 0.3M NaCl linear  
gradient. Further purification was by chromatography on a  
Blue Toyopearl® column.

F. Reticulocyte Lysate Assay

The ability of the rBRIP and the BRIP analogs to  
inhibit protein synthesis in vitro was tested by  
reticulocyte lysate assay as described in Example 1.  
Serial log dilutions of standard toxin (RTA 30), native  
BRIP, rBRIP and BRIP analogs were tested over a range of 1  
 $\mu$ g/ml to 1 pg/ml. By comparison with an uninhibited  
sample, the picomolar concentration of toxin (pM) which  
corresponds to 50% inhibition of protein synthesis ( $IC_{50}$ )  
was calculated. The results of the assays are presented  
below in Table 17.

-136-

Table 17

	<u>Toxin</u>	<u>IC<sub>50</sub> (pM)</u>
	RTA 30	3.1
	Native BRIP	15
5	rBRIP	18
	BRIP <sub>C256</sub>	23
	BRIP <sub>C270</sub>	20
	BRIP <sub>C277</sub>	24

10 The RLA results indicate that the BRIP analogs exhibit ribosome-inactivating activity comparable to that of the recombinant and native BRIP toxin. All the analogs retained the natural ability of native BRIP to inhibit protein synthesis, suggesting that amino acid substitution at these positions does not affect protein folding and  
15 activity.

Example 23Construction Of BRIP Immunoconjugates

Immunoconjugates of native BRIP (SEQ ID NO: 3) with 4A2 (described in Morishima et al., *J. Immunol.*, 129:1091 (1982) and H65 antibody (obtained from hybridoma ATCC HB9286) which recognize the T-cell determinants CD7 and CD5, respectively, were constructed. Immunoconjugates of ricin A-chains (RTAs) with 4A2 and H65 antibody were constructed as controls. The H65 antibody and ricin A-chains as well as the RTA immunoconjugates were prepared and purified according to methods described in U.S. Patent Application Serial No. 07/306,433 *supra* and in International Publication No. WO 89/06968.

30 To prepare immunoconjugates of native BRIP, both the antibody (4A2 or H65) and native BRIP were chemically modified with the hindered linker 5-methyl-2-iminothiolane (M2IT) at lysine residues to introduce a reactive sulfhydryl group as described in Goff et al., *Bioconjugate Chem.*, 1:381-386 (1990). BRIP (3 mg/ml) was first  
35 incubated with 0.5 mM M2IT and 1 mM DTNB in 25 mM

-137-

triethanolamine, 150 mM NaCl, pH 8.0, for 3 hours at 25°C. The derivitized BRIP-(M2IT)-S-S-TNB was then desalted on a column of Sephadex GF-05LS and the number of thiol groups introduced was quantitated by the addition of 0.1 mM DTT. On average, each BRIP molecule contained 0.7 SH/mol.

4A2 or H65 antibody (4 mg/ml) in triethanolamine buffer was similarly incubated with M2IT (0.3 mM) and DTNB (1 mM) for 3 hours at 25°C. Antibody-(M2IT)-S-S-TNB was then desalted and the TNB:antibody ratio was determined.

To prepare the conjugate, the BRIP-(M2IT)-S-S-TNB was first reduced to BRIP-(M2IT)-SH by treatment with 0.5 mM DTT for 1 hour at 25°C, desalted by gel filtration of Sephadex® GF-05LS to remove the reducing agent, and then mixed with antibody-(M2IT)-S-S-TNB.

Following a 3 hour incubation at 25°C, and an additional 18 hours at 4°C, the conjugate was purified by sequential chromatography on Aca44 (IBF) and Blue Toyopearl®. Samples of the final product were run on 5% non-reducing SDS PAGE, Coomassie stained, and scanned with a Shimadzu laser densitometer to quantitate the number of toxins per antibody.

The BRIP analogs containing a free cysteine were also conjugated to 4A2 and H65 antibodies. The analogs were treated with 50 mM DTT either for 2 hours at 25°C or for 18 hours at 4°C to expose the reactive sulfhydryl group of the cysteine and desalted. The presence of a free sulfhydryl was verified by reaction with DTNB [Ellman et al., *Arch. Biochem. Biophys.*, 82:70-77 (1959)]. 4A2 or H65 antibody derivitized as described above with M2IT was incubated with the reduced BRIP analogs at a ratio of 1:5 at room temperature for 3 hours and then overnight at 4°C. Immunoconjugates H65-BRIP<sub>C256</sub>, 4A2-BRIP<sub>C256</sub>, H65-BRIP<sub>C277</sub> were prepared in 25 mM triethanolamine, 150 mM NaCl pH 8, while immunoconjugates H65-BRIP<sub>C270</sub>, 4A2-BRIP<sub>C270</sub> and 4A2-BRIP<sub>C277</sub> were prepared in 0.1 M sodium phosphate, 150 mM NaCl pH 7.5. Following conjugation, 10 µM mercaptoethylamine was added for 15 minutes at 25°C to quench any unreacted m2IT

-138-

linkers on the antibody. The quenched reaction solution was promptly loaded onto a gel filtration column (AcA44) to ~~remove—unconjugated—ribosome-inactivating—~~ protein. Purification was completed using soft gel affinity chromatography on Blue Toyopearl® resin using a method similar to Knowles et al., *Analyt. Biochem.*, 160:440 (1987). Samples of the final product were run on 5% non-reduced SDS PAGE, Coomassie stained, and scanned with a Shimadzu laser densitometer to quantitate the number of toxins per antibody. The conjugation efficiency was substantially greater for BRIP<sub>C277</sub> (78%) than for either of the other two analogs, BRIP<sub>C270</sub> and BRIP<sub>C256</sub> (each of these was about 10%). Additionally, the BRIP<sub>C277</sub> product was a polyconjugate, i.e., several BRIP molecules conjugated to a single antibody, in contrast to the BRIP<sub>C270</sub> and BRIP<sub>C256</sub> products which were monoconjugates.

#### Example 24

##### Properties Of BRIP Immunoconjugates

##### A. Whole Cell Kill Assay

Immunoconjugates of native BRIP and of the BRIP analogs were tested for the ability to inhibit protein synthesis in HSB2 cells by the whole cell kill assay described in Example 1. Standard immunoconjugates H65-RTA (H65 derivatized with SPDP linked to RTA) and 4MRTA (4A2 antibody derivatized with M2IT linked to RTA) and BRIP immunoconjugate samples were diluted with RPMI without leucine at half-log concentrations ranging from 2000 to 0.632 ng/ml. All dilutions were added in triplicate to microtiter plates containing  $1 \times 10^5$  HSB2 cells. HSB2 plates were incubated for 20 hours at 37°C and then pulsed with <sup>3</sup>H-Leu for 4 hours before harvesting. Samples were counted on the Inotec Trace 96 cascade ionization counter. By comparison with an untreated sample, the picomolar toxin concentration (pM T) of immunoconjugate which resulted in a 50% inhibition of protein synthesis (IC<sub>50</sub>) was calculated. The assay results are presented below in Table 18.

-139-

Table 18

	<u>Conjugate</u>	<u>IC<sub>50</sub> (pM T)</u>
	4A2-BRIP	122.45
	4A2-BRIP <sub>C270</sub>	46.3
5	4A2-BRIP <sub>C277</sub>	57.5
	4A2-BRIP <sub>C256</sub>	1116
	H65-BRIP	>5000
	H65-BRIP <sub>C277</sub>	1176

10       The BRIP analog conjugates were less potent than  
 the ricin conjugate control (data not shown). The  
 immunotoxins containing antibody 4A2 and either the BRIP<sub>C270</sub>  
 or the BRIP<sub>C277</sub> analog exhibited comparable to increased  
 specific cytotoxicity toward target cells as compared to  
 immunotoxin containing native BRIP. While 4A2-BRIP<sub>C256</sub> is  
 15       less active than 4A2-BRIP, 4A2-BRIP<sub>C270</sub> and 4A2-BRIP<sub>C277</sub> are  
 between 3 and 4 times more active. Similarly, the  
 immunoconjugate of H65 to BRIP<sub>C277</sub> shows greater toxicity  
 toward target cells than the immunoconjugate of H65 to  
 native BRIP. Thus, linkage of antibody to BRIP derivatives  
 20       which have an available cysteine residue in an appropriate  
 location results in immunotoxins with enhanced specific  
 toxicity toward target cells relative to conjugates with  
 native BRIP.

#### B. Disulfide Bond Stability Assay

25       Immunoconjugates prepared with native BRIP and  
 the BRIP analogs were examined by the disulfide bond  
 stability assay described in Example 1. Briefly,  
 conjugates were incubated with increasing concentrations of  
 glutathione for 1 hour at 37°C and, after terminating the  
 30       reaction with iodoacetamide, the amount of RIP released was  
 quantitated by size-exclusion HPLC on a TosohHaas TSK-  
 G2000SW column.

By comparisons with the amount of RIP released by  
 high concentrations of 2-mercaptoethanol (to determine 100%  
 35       release), the concentration of glutathione required to

-140-

release 50% of the RIP (the  $RC_{50}$ ) was calculated. As shown below in Table 19, the conjugates prepared with BRIP<sub>C270</sub> or BRIP<sub>C277</sub> were significantly more stable than either the RTA conjugates or those prepared with native BRIP.

5

Table 19

	<u>Conjugate</u>	<u>RC<sub>50</sub> (mM)</u>
	H65-RTA	7.0
	H65-BRIP	2.8
	H65-BRIPC277	196.0
10	4A2-RTA	4.4
	4A2-BRIP	3.3
	42-BRIP <sub>C270</sub>	53.0
	4A2-BRIP <sub>C277</sub>	187.0

These unexpected results suggest that conjugates prepared with Type I RIP analogs according to the present invention may have enhanced stability and efficacy *in vivo*.

15

Example 25Preparation of Momordin and Analogs Thereof

Plants of the genus *Momordica* produce a number of related proteins known as momordins or momorcharins which are Type I RIPs. The gene encoding momordin II was cloned from *Momordica balsamina* seeds.

20

A. Preparation Of *M. balsamina* RNA

Total RNA was prepared from 4 g of *M. balsamina* seeds as described in Ausubel et al., *supra*. PolyA containing RNA was prepared from 1 mg of total RNA by chromatography on oligo-(dT)-cellulose. 40 mg of oligo-(dT)-cellulose Type 7 (Pharmacia) was added to 0.1 N NaOH and poured into a disposable column (Biorad). The column was washed with water until the eluate was pH 5.5, and then was washed with 1X loading buffer (50 mM NaCitate, 0.5M NaCl, 1 mM EDTA, 0.1% SDS, pH 7.0) until the eluate was pH 7.0. 1 mg of total RNA was suspended in 300  $\mu$ l of water,

25

30



-141-

heated to 65°C for 5 minutes, and 300 µl of 2X loading buffer was added (100 mM Na Citrate, 1M NaCl, 2 mM EDTA, and 0.2% SDS). The RNA was loaded onto the column, and the flow through was reheated to 65°C, cooled to room temperature, and reloaded onto the column. Column-bound mRNA was washed 5 times with 0.5 ml of 1X loading buffer, and two times with 0.5 ml of 0.05M NaCitrate, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS. PolyA- containing RNA was eluted two times from the column with 0.5 ml of 25 mM NaCitrate, 1 mM EDTA, and 0.05% SDS.

#### B. Library Preparation

A cDNA library from the polyA-containing *M. balsamina* RNA was prepared in a bacterial expression plasmid with the SuperScript Plasmid System (BRL, Gaithersburg, Maryland). The cDNA was synthesized from 2 µg of poly A-containing RNA, size fractionated, digested with NotI, and ligated into the expression vector pSPORT as recommended by the manufacturer of the vector, BRL.

#### C. Cloning Of The Momordin II Gene

A DNA fragment encoding the first 27 amino acids of momordin II was amplified from *M. balsamina* cDNA by PCR. First strand cDNA was prepared from 100 ng of polyA containing RNA with an RNA-PCR Kit (Perkin Elmer Cetus). Two partially degenerate primers were synthesized based on the amino acid sequence of the first 27 amino acids of momordin II described in Li et al., *Experientia*, 36:524-527 (1980). Because the amino acid sequence of amino acids 1-27 of momordin II is 52% homologous to amino acids 1-17 of momordin I [Ho et al., *BBA*, 1088:311-314 (1991)], some codon assignments in the degenerate primers were based on homology to the corresponding amino acid as well as codon preference in the momordin I gene. The sequences of primers momo-3 and momo-4 are set out below using IUPAC nucleotide symbols.

-142-

momo-3 (SEQ ID NO: 51)

5' GATGTTAAYTTYGAYTTGTCNACDGCTAC 3'

momo-4 (SEQ ID NO: 52)

5' ATTGGNAGDGTAGCCCTRAARTCYTCDAT 3'

5 The resulting 81 bp PCR product was purified on a 5% acrylamide gel and cloned into the *Sma*I site of pUC18. Three candidate clones were sequenced, and one clone, pMO110, was identified which encoded the N-terminal 27 amino acids of momordin II.

10 A hybridization probe was designed for screening of the momordin II cDNA library based on the sequence of the pMO110 momordin II DNA fragment. The sequence of the primer momo-5 is shown below.

momo-5 (SEQ ID NO: 53)

15 5' GCCACTGCAAAAACCTACACAAAATTTATTGA 3'

Primer momo-5 corresponds to amino acids 9-18 of mature momordin II. The underlined nucleotides of the primer were expected to match the DNA sequence of the momordin II gene exactly. Since this sequence is highly A/T-rich and may hybridize to the momordin II gene weakly, the additional adjacent nucleotides were included in the primer. Bases 3 and 30 (overlined) were in the "wobble" position (i.e., the third nucleotide in a codon) of amino acids 9 (alanine) and 18 (isoleucine), respectively, of momordin II and may not be identical to the nucleotide bases in the native gene.

20 A 90,000 member cDNA library in pSPORT was screened with <sup>32</sup>P-kinased momo-5, and eight potential candidate clones were identified. One clone, pING3619, was sequenced and contains an open reading frame corresponding in part to the expected N-terminal 27 residues of Momordin II. The complete momordin gene contains 286 amino acids, the first 23 of which are a presumed leader signal (mature momordin II is 263 residues). The DNA sequence of the momordin II gene is set out in SEQ ID NO: 13.

-143-

D. Construction Of An Expression  
Vector Containing The Momordin II Gene

~~A bacterial expression vector for the momordin II~~  
gene was constructed. Two PCR primers were synthesized,  
one (momo-9) which primes from the +1 residue of the mature  
momordin II amino acid sequence, and one at the C-terminus  
(momo-10) of momordin II which introduces an *XhoI*  
restriction site:

momo-9 (SEQ ID NO: 54)

5' GATGTAACTTCGATTTGTCTGA 3'

momo-10 (SEQ ID NO: 55)

5' TCAACTCGAGGTACTCAATTCACAACAGATTCC 3'

pING3619 was amplified with momo-9 and momo-10, and the  
product was treated with T4 polymerase, cut with *XhoI*, and  
purified on an agarose gel. This gene fragment was ligated  
along with the 131 bp *pelB* leader fragment from pIC100  
which has been generated by *SstI* digestion, T4-polymerase  
treatment, and *EcoRI* digestion, into the *araB* expression  
vector cleaved with *EcoRI* and *XhoI*. The product of this  
three piece ligation was sequenced to verify that the *pelB*  
junction and momordin II coding sequence were correct.  
Arabinose induction of cells containing the momordin II  
expression plasmid pING3621 results in production of  
momordin II in *E. coli*.

E. Analogues Of Mormordin II

Mormordin II has no natural cysteines available  
for conjugation to antibody. Analogs of momordin which  
have a free cysteine for conjugation to an antibody may be  
constructed. Positions likely to be appropriate for  
substitution of a cysteine residue may be identified from  
Figure 3 as positions near the ricin A-chain cysteine<sub>259</sub>, and  
as positions including the last 26 amino acids of momordin  
II that are accessible to solvent. For example, the  
arginine at position 242 of momordin II aligns with the  
ricin A-chain cysteine at position 259 and is a preferred  
target for substitution. Additional preferred substitution

-144-

positions for momordin II include the serine at position 241 and the alanine at position 243.

5 While the present invention has been described in terms of preferred embodiments, it is understood that variations and improvements will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

-145-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Xoma Corporation
- (ii) TITLE OF INVENTION: Immunotoxins Comprising Ribosome-Inactivating Proteins
- (iii) NUMBER OF SEQUENCES: 144
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
  - (B) STREET: 6300 Sears Tower, 233 South Wacker Drive
  - (C) CITY: Chicago
  - (D) STATE: Illinois
  - (E) COUNTRY: USA
  - (F) ZIP: 60606-6402
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/064,691
  - (B) FILING DATE: 12-MAY-1993
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 07/988,430
  - (B) FILING DATE: 09-DEC-1992
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT US92/09487
  - (B) FILING DATE: 04-NOV-1992
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 07/901,707
  - (B) FILING DATE: 19-JUN-1992
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 07/787,567
  - (B) FILING DATE: 04-NOV-1991
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Noland, Greta E.
  - (B) REGISTRATION NUMBER: 35,302
  - (C) REFERENCE/DOCKET NUMBER: 32088
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 312/474-6300
  - (B) TELEFAX: 312/474-0448
  - (C) TELEX: 25-3856

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 267 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

-146-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Ile Phe Pro Lys Gln Tyr Pro Ile Ile Asn Phe Thr Thr Ala Gly Ala
1           5           10           15
Thr Val Gln Ser Tyr Thr Asn Phe Ile Arg Ala Val Arg Gly Arg Leu
20           25           30
Thr Thr Gly Ala Asp Val Arg His Glu Ile Pro Val Leu Pro Asn Arg
35           40           45
Val Gly Leu Pro Ile Asn Gln Arg Phe Ile Leu Val Glu Leu Ser Asn
50           55           60
His Ala Glu Leu Ser Val Thr Leu Ala Leu Asp Val Thr Asn Ala Tyr
65           70           75           80
Val Val Gly Tyr Arg Ala Gly Asn Ser Ala Tyr Phe Phe His Pro Asp
85           90           95
Asn Gln Glu Asp Ala Glu Ala Ile Thr His Leu Phe Thr Asp Val Gln
100          105          110
Asn Arg Tyr Thr Phe Ala Phe Gly Gly Asn Tyr Asp Arg Leu Glu Gln
115          120          125
Leu Ala Gly Asn Leu Arg Glu Asn Ile Glu Leu Gly Asn Gly Pro Leu
130          135          140
Glu Glu Ala Ile Ser Ala Leu Tyr Tyr Tyr Ser Thr Gly Gly Thr Gln
145          150          155          160
Leu Pro Thr Leu Ala Arg Ser Phe Ile Ile Cys Ile Gln Met Ile Ser
165          170          175
Glu Ala Ala Arg Phe Gln Tyr Ile Glu Gly Glu Met Arg Thr Arg Ile
180          185          190
Arg Tyr Asn Arg Arg Ser Ala Pro Asp Pro Ser Val Ile Thr Leu Glu
195          200          205
Asn Ser Trp Gly Arg Leu Ser Thr Ala Ile Gln Glu Ser Asn Gln Gly
210          215          220
Ala Phe Ala Ser Pro Ile Gln Leu Gln Arg Arg Asn Gly Ser Lys Phe
225          230          235          240
Ser Val Tyr Asp Val Ser Ile Leu Ile Pro Ile Ile Ala Leu Met Val
245          250          255
Tyr Arg Cys Ala Pro Pro Pro Ser Ser Gln Phe
260          265

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 251 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

-147-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gly	Leu	Asp	Thr	Val	Ser	Phe	Ser	Thr	Lys	Gly	Ala	Thr	Tyr	Ile	Thr
1				5					10						15
Tyr	Val	Asn	Phe	Leu	Asn	Glu	Leu	Arg	Val	Lys	Leu	Lys	Pro	Glu	Gly
			20					25					30		
Asn	Ser	His	Gly	Ile	Pro	Leu	Leu	Arg	Lys	Lys	Cys	Asp	Asp	Pro	Gly
		35					40					45			
Lys	Cys	Phe	Val	Leu	Val	Ala	Leu	Ser	Asn	Asp	Asn	Gly	Gln	Leu	Ala
	50					55					60				
Glu	Ile	Ala	Ile	Asp	Val	Thr	Ser	Val	Tyr	Val	Val	Gly	Tyr	Gln	Val
65					70					75					80
Arg	Asn	Arg	Ser	Tyr	Phe	Phe	Lys	Asp	Ala	Pro	Asp	Ala	Ala	Tyr	Glu
				85					90					95	
Gly	Leu	Phe	Lys	Asn	Thr	Ile	Lys	Thr	Arg	Leu	His	Phe	Gly	Gly	Ser
			100					105					110		
Tyr	Pro	Ser	Leu	Glu	Gly	Glu	Lys	Ala	Tyr	Arg	Glu	Thr	Thr	Asp	Leu
		115					120					125			
Gly	Ile	Glu	Pro	Leu	Arg	Ile	Gly	Ile	Lys	Lys	Leu	Asp	Glu	Asn	Ala
	130					135					140				
Ile	Asp	Asn	Tyr	Lys	Pro	Thr	Glu	Ile	Ala	Ser	Ser	Leu	Leu	Val	Val
145					150					155					160
Ile	Gln	Met	Val	Ser	Glu	Ala	Ala	Arg	Phe	Thr	Phe	Ile	Glu	Asn	Gln
				165					170					175	
Ile	Arg	Asn	Asn	Phe	Gln	Gln	Arg	Ile	Arg	Pro	Ala	Asn	Asn	Thr	Ile
			180					185					190		
Ser	Leu	Glu	Asn	Lys	Trp	Gly	Lys	Leu	Ser	Phe	Gln	Ile	Arg	Thr	Ser
		195					200					205			
Gly	Ala	Asn	Gly	Met	Phe	Ser	Glu	Ala	Val	Glu	Leu	Glu	Arg	Ala	Asn
	210					215					220				
Gly	Lys	Lys	Tyr	Tyr	Val	Thr	Ala	Val	Asp	Gln	Val	Lys	Pro	Lys	Ile
225					230					235					240
Ala	Leu	Leu	Lys	Phe	Val	Asp	Lys	Asp	Pro	Lys					
				245					250						

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Ala Lys Met Ala Lys Asn Val Asp Lys Pro Leu Phe Thr Ala Thr  
1 5 10 15

-148-

Phe Asn Val Gln Ala Ser Ser Ala Asp Tyr Ala Thr Phe Ile Ala Gly  
                   20                  25                  30  
 Ile Arg Asn Lys Leu Arg Asn Pro Ala His Phe Ser His Asn Arg Pro  
           35                  40                  45  
 Val Leu Pro Pro Val Glu Pro Asn Val Pro Pro Ser Arg Trp Phe His  
           50                  55                  60  
 Val Val Leu Lys Ala Ser Pro Thr Ser Ala Gly Leu Thr Leu Ala Ile  
           65                  70                  75                  80  
 Arg Ala Asp Asn Ile Tyr Leu Glu Gly Phe Lys Ser Ser Asp Gly Thr  
                   85                  90                  95  
 Trp Trp Glu Leu Thr Pro Gly Leu Ile Pro Gly Ala Thr Tyr Val Gly  
                   100                  105                  110  
 Phe Gly Gly Thr Tyr Arg Asp Leu Leu Gly Asp Thr Asp Lys Leu Thr  
                   115                  120                  125  
 Asn Val Ala Leu Gly Arg Gln Gln Leu Ala Asp Ala Val Thr Ala Leu  
           130                  135                  140  
 His Gly Arg Thr Lys Ala Asp Lys Ala Ser Gly Pro Lys Gln Gln Gln  
           145                  150                  155                  160  
 Ala Arg Glu Ala Val Thr Thr Leu Val Leu Met Val Asn Glu Ala Thr  
                   165                  170                  175  
 Arg Phe Gln Thr Val Ser Gly Phe Val Ala Gly Leu Leu His Pro Lys  
                   180                  185                  190  
 Ala Val Glu Lys Lys Ser Gly Lys Ile Gly Asn Glu Met Lys Ala Gln  
           195                  200                  205  
 Val Asn Gly Trp Gln Asp Leu Ser Ala Ala Leu Leu Lys Thr Asp Val  
           210                  215                  220  
 Lys Pro Pro Pro Gly Lys Ser Pro Ala Lys Phe Ala Pro Ile Glu Lys  
           225                  230                  235                  240  
 Met Gly Val Arg Thr Ala Glu Gln Ala Ala Asn Thr Leu Gly Ile Leu  
                   245                  250                  255  
 Leu Phe Val Glu Val Pro Gly Gly Leu Thr Val Ala Lys Ala Leu Glu  
           260                  265                  270  
 Leu Phe His Ala Ser Gly Gly Lys  
           275                  280

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
     (A) LENGTH: 263 amino acids  
     (B) TYPE: amino acid  
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Val Asn Phe Asp Leu Ser Thr Ala Thr Ala Lys Thr Tyr Thr Lys  
   1                  5                  10                  15



-149-

Phe Ile Glu Asp Phe Arg Ala Thr Leu Pro Phe Ser His Lys Val Tyr  
                     20                    25                    30  
~~---Asp-Ile-Pro-Leu-Leu-Tyr-Ser-Thr-Ile-Ser-Asp-Ser-Arg Arg Phe Ile~~  
                     35                    40                    45  
 Leu Leu Asp Leu Thr Ser Tyr Ala Tyr Glu Thr Ile Ser Val Ala Ile  
           50                    55                    60  
 Asp Val Thr Asn Val Tyr Val Val Ala Tyr Arg Thr Arg Asp Val Ser  
   65                    70                    75                    80  
 Tyr Phe Phe Lys Glu Ser Pro Pro Glu Ala Tyr Asn Ile Leu Phe Lys  
                     85                    90                    95  
 Gly Thr Arg Lys Ile Thr Leu Pro Tyr Thr Gly Asn Tyr Glu Asn Leu  
                     100                    105                    110  
 Gln Thr Ala Ala His Lys Ile Arg Glu Asn Ile Asp Leu Gly Leu Pro  
           115                    120                    125  
 Ala Leu Ser Ser Ala Ile Thr Thr Leu Phe Tyr Tyr Asn Ala Gln Ser  
   130                    135                    140  
 Ala Pro Ser Ala Leu Leu Val Leu Ile Gln Thr Thr Ala Glu Ala Ala  
  145                    150                    155                    160  
 Arg Phe Lys Tyr Ile Glu Arg His Val Ala Lys Tyr Val Ala Thr Asn  
           165                    170                    175  
 Phe Lys Pro Asn Leu Ala Ile Ile Ser Leu Glu Asn Gln Trp Ser Ala  
           180                    185                    190  
 Leu Ser Lys Gln Ile Phe Leu Ala Gln Asn Gln Gly Gly Lys Phe Arg  
           195                    200                    205  
 Asn Pro Val Asp Leu Ile Lys Pro Thr Gly Glu Arg Phe Gln Val Thr  
   210                    215                    220  
 Asn Val Asp Ser Asp Val Val Lys Gly Asn Ile Lys Leu Leu Leu Asn  
  225                    230                    235                    240  
 Ser Arg Ala Ser Thr Ala Asp Glu Asn Phe Ile Thr Thr Met Thr Leu  
           245                    250                    255  
 Leu Gly Glu Ser Val Val Asn  
           260

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 248 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Val Arg Phe Ser Leu Ser Gly Ser Ser Ser Thr Ser Tyr Ser Lys  
   1                    5                    10                    15  
 Phe Ile Gly Asp Leu Arg Lys Ala Leu Pro Ser Asn Gly Thr Val Tyr  
           20                    25                    30

-150-

```

Asn Leu Thr Ile Leu Leu Ser Ser Ala Ser Gly Ala Ser Arg Tyr Thr
 35                      40                      45
Leu Met Thr Leu Ser Asn Tyr Asp Gly Lys Ala Ile Thr Val Ala Val
 50                      55                      60
Asp Val Ser Gln Leu Tyr Ile Met Gly Tyr Leu Val Asn Ser Thr Ser
 65                      70                      75                      80
Tyr Phe Phe Asn Glu Ser Asp Ala Lys Leu Ala Ser Gln Tyr Val Phe
                      85                      90                      95
Lys Gly Ser Thr Ile Val Thr Leu Pro Tyr Ser Gly Asn Tyr Glu Lys
                      100                      105                      110
Leu Gln Thr Ala Ala Gly Lys Ile Arg Glu Lys Ile Pro Leu Gly Phe
                      115                      120                      125
Pro Ala Leu Asp Ser Ala Leu Thr Thr Ile Phe His Tyr Asp Ser Thr
                      130                      135                      140
Ala Ala Ala Ala Ala Phe Leu Val Ile Leu Gln Thr Thr Ala Glu Ala
                      145                      150                      155                      160
Ser Arg Phe Lys Tyr Ile Glu Gly Gln Ile Ile Glu Arg Ile Ser Lys
                      165                      170                      175
Asn Gln Val Pro Ser Leu Ala Thr Ile Ser Leu Glu Asn Ser Leu Trp
                      180                      185                      190
Ser Ala Leu Ser Lys Gln Ile Gln Leu Ala Gln Thr Asn Asn Gly Thr
                      195                      200                      205
Phe Lys Thr Pro Val Val Ile Thr Asp Asp Lys Gly Gln Arg Val Glu
                      210                      215                      220
Ile Thr Asn Val Thr Ser Lys Val Val Thr Lys Asn Ile Gln Leu Leu
                      225                      230                      235                      240
Leu Asn Tyr Lys Gln Asn Val Ala
                      245

```

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 255 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Asp Val Ser Phe Arg Leu Ser Gly Ala Thr Ser Ser Ser Tyr Gly Val
 1                      5                      10                      15
Phe Ile Ser Asn Leu Arg Lys Ala Leu Pro Asn Glu Arg Lys Leu Tyr
                      20                      25                      30
Asp Leu Pro Leu Ile Arg Ser Ser Leu Pro Gly Ser Gln Arg Tyr Ala
                      35                      40                      45
Ile Ile His Leu Thr Asn Tyr Ala Asp Glu Val Ala Leu Asp Val Thr
                      50                      55                      60

```

Asn	Val	Asp	Ala	Gly	Leu	Pro	Arg	Asn	Ala	Val	Leu	Tyr	Ile	Met	Gly
65					70					75					80
Tyr	Arg	Ala	Gly	Asp	Thr	Ser	Tyr	Phe	Phe	Asn	Glu	Ala	Ser	Ala	Thr
				85					90					95	
Glu	Ala	Ala	Lys	Tyr	Val	Phe	Lys	Asp	Ala	Met	Arg	Lys	Val	Thr	Leu
			100					105					110		
Pro	Tyr	Ser	Gly	Asn	Tyr	Glu	Arg	Leu	Gln	Thr	Ala	Ala	Gly	Gly	Leu
		115					120					125			
Arg	Glu	Asn	Ile	Pro	Leu	Gly	Leu	Pro	Ala	Leu	Asp	Ser	Ala	Ile	Thr
	130					135					140				
Thr	Leu	Phe	Tyr	Tyr	Asn	Ala	Asn	Ser	Ala	Ala	Ser	Ala	Leu	Met	Val
145					150					155					160
Leu	Ile	Gln	Ser	Thr	Ser	Glu	Ala	Ala	Arg	Tyr	Lys	Phe	Ile	Glu	Gln
				165					170					175	
Gln	Ile	Gly	Ser	Arg	Val	Asp	Lys	Thr	Phe	Leu	Pro	Ser	Leu	Ala	Ile
			180					185					190		
Ile	Ser	Leu	Glu	Asn	Ser	Leu	Trp	Leu	Ala	Leu	Ser	Lys	Gln	Ile	Gln
		195					200					205			
Ile	Ala	Ser	Thr	Asn	Asn	Gly	Glu	Phe	Glu	Thr	Pro	Val	Val	Leu	Ile
	210					215					220				
Asn	Ala	Gln	Asn	Gln	Arg	Val	Thr	Ile	Thr	Asn	Val	Asp	Ala	Gly	Val
225					230					235					240
Val	Thr	Ser	Asn	Ile	Ala	Leu	Leu	Leu	Asn	Arg	Asn	Asn	Met	Ala	
				245					250					255	

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 263 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asp 1	Val	Ser	Phe	Arg 5	Leu	Ser	Gly	Ala	Asp 10	Pro	Arg	Ser	Tyr	Gly 15	Met
Phe	Ile	Lys	Asp 20	Leu	Arg	Asn	Ala	Leu 25	Pro	Phe	Arg	Glu	Lys 30	Val	Tyr
Asn	Ile	Pro 35	Leu	Leu	Leu	Pro	Ser 40	Val	Ser	Gly	Ala	Gly 45	Arg	Tyr	Leu
Leu	Met 50	His	Leu	Phe	Asn	Tyr 55	Asp	Gly	Lys	Thr	Ile 60	Thr	Val	Ala	Val
Asp 65	Val	Thr	Asn	Val	Tyr 70	Ile	Met	Gly	Tyr	Leu 75	Ala	Asp	Thr	Thr	Ser 80
Tyr	Phe	Phe	Asn	Glu 85	Pro	Ala	Ala	Glu	Leu 90	Ala	Ser	Gln	Tyr	Val 95	Phe

-152-

Arg Asp Ala Arg Arg Lys Ile Thr Leu Pro Tyr Ser Gly Asn Tyr Glu  
 100 105 110  
 Arg Leu Gln Ile Ala Ala Gly Lys Pro Arg Glu Lys Ile Pro Ile Gly  
 115 120 125  
 Leu Pro Ala Leu Asp Ser Ala Ile Ser Thr Leu Leu His Tyr Asp Ser  
 130 135 140  
 Thr Ala Ala Ala Gly Ala Leu Leu Val Leu Ile Gln Thr Thr Ala Glu  
 145 150 155 160  
 Ala Ala Arg Phe Lys Tyr Ile Glu Gln Gln Ile Gln Glu Arg Ala Tyr  
 165 170 175  
 Arg Asp Glu Val Pro Ser Leu Ala Thr Ile Ser Leu Glu Asn Ser Trp  
 180 185 190  
 Ser Gly Leu Ser Lys Gln Ile Gln Leu Ala Gln Gly Asn Asn Gly Ile  
 195 200 205  
 Phe Arg Thr Pro Ile Val Leu Val Asp Asn Lys Gly Asn Arg Val Gln  
 210 215 220  
 Ile Thr Asn Val Thr Ser Lys Val Val Thr Ser Asn Ile Gln Leu Leu  
 225 230 235 240  
 Leu Asn Thr Arg Asn Ile Ala Glu Gly Asp Asn Gly Asp Val Ser Thr  
 245 250 255  
 Thr His Gly Phe Ser Ser Thr  
 260

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 250 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Pro Thr Leu Glu Thr Ile Ala Ser Leu Asp Leu Asn Asn Pro Thr  
 1 5 10 15  
 Thr Tyr Leu Ser Phe Ile Thr Asn Ile Arg Thr Lys Val Ala Asp Lys  
 20 25 30  
 Thr Glu Gln Cys Thr Ile Gln Lys Ile Ser Lys Thr Phe Thr Gln Arg  
 35 40 45  
 Tyr Ser Tyr Ile Asp Leu Ile Val Ser Ser Thr Gln Lys Ile Thr Leu  
 50 55 60  
 Ala Ile Asp Met Ala Asp Leu Tyr Val Leu Gly Tyr Ser Asp Ile Ala  
 65 70 75 80  
 Asn Asn Lys Gly Arg Ala Phe Phe Phe Lys Asp Val Thr Glu Ala Val  
 85 90 95  
 Ala Asn Asn Phe Phe Pro Gly Ala Thr Gly Thr Asn Arg Ile Lys Leu  
 100 105 110

-153-

Thr Phe Thr Gly Ser Tyr Gly Asp Leu Glu Lys Asn Gly Gly Leu Arg  
 115 120 125  
~~Lys Asp Asn Pro Leu Gly Ile Phe Arg Leu Glu Asn Ser Ile Val Asn~~  
 130 135 140  
 Ile Tyr Gly Lys Ala Gly Asp Val Lys Lys Gln Ala Lys Phe Phe Leu  
 145 150 155 160  
 Leu Ala Ile Gln Met Val Ser Glu Ala Ala Arg Phe Lys Tyr Ile Ser  
 165 170 175  
 Asp Lys Ile Pro Ser Glu Lys Tyr Glu Glu Val Thr Val Asp Glu Tyr  
 180 185 190  
 Met Thr Ala Leu Glu Asn Asn Trp Ala Lys Leu Ser Thr Ala Val Tyr  
 195 200 205  
 Asn Ser Lys Pro Ser Thr Thr Thr Ala Thr Lys Cys Gln Leu Ala Thr  
 210 215 220  
 Ser Pro Val Thr Ile Ser Pro Trp Ile Phe Lys Thr Val Glu Glu Ile  
 225 230 235 240  
 Lys Leu Val Met Gly Leu Leu Lys Ser Ser  
 245 250

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 261 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ile Asn Thr Ile Thr Phe Asp Ala Gly Asn Ala Thr Ile Asn Lys Tyr  
 1 5 10 15  
 Ala Thr Phe Met Glu Ser Leu Arg Asn Glu Ala Lys Asp Pro Ser Leu  
 20 25 30  
 Lys Cys Tyr Gly Ile Pro Met Leu Pro Asn Thr Asn Ser Thr Ile Lys  
 35 40 45  
 Tyr Leu Leu Val Lys Leu Gln Gly Ala Ser Leu Lys Thr Ile Thr Leu  
 50 55 60  
 Met Leu Arg Arg Asn Asn Leu Tyr Val Met Gly Tyr Ser Asp Pro Tyr  
 65 70 75 80  
 Asp Asn Lys Cys Arg Tyr His Ile Phe Asn Asp Ile Lys Gly Thr Glu  
 85 90 95  
 Tyr Ser Asp Val Glu Asn Thr Leu Cys Pro Ser Ser Asn Pro Arg Val  
 100 105 110  
 Ala Lys Pro Ile Asn Tyr Asn Gly Leu Tyr Pro Thr Leu Glu Lys Lys  
 115 120 125  
 Ala Gly Val Thr Ser Arg Asn Glu Val Gln Leu Gly Ile Gln Ile Leu  
 130 135 140

-154-

Ser Ser Lys Ile Gly Lys Ile Ser Gly Gln Gly Ser Phe Thr Glu Lys  
 145 150 155 160  
 Ile Glu Ala Asp Phe Leu Leu Val Ala Ile Gln Met Val Ser Glu Ala  
 165 170 175  
 Ala Arg Phe Lys Tyr Ile Glu Asn Gln Val Lys Thr Asn Phe Asn Arg  
 180 185 190  
 Asp Phe Ser Pro Asn Asp Lys Val Leu Asp Leu Glu Glu Asn Trp Gly  
 195 200 205  
 Lys Ile Ser Thr Ala Ile His Asn Ser Lys Asn Gly Ala Leu Pro Lys  
 210 215 220  
 Pro Leu Glu Leu Lys Asn Ala Asp Gly Thr Lys Trp Ile Val Leu Arg  
 225 230 235 240  
 Val Asp Glu Ile Lys Pro Asp Val Gly Leu Leu Asn Tyr Val Asn Gly  
 245 250 255  
 Thr Cys Gln Ala Thr  
 260

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 259 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Thr Ser Ile Thr Leu Asp Leu Val Asn Pro Thr Ala Gly Gln Tyr  
 1 5 10 15  
 Ser Ser Phe Val Asp Lys Ile Arg Asn Asn Val Lys Asp Pro Asn Leu  
 20 25 30  
 Lys Tyr Gly Gly Thr Asp Ile Ala Val Ile Gly Pro Pro Ser Lys Glu  
 35 40 45  
 Lys Phe Leu Arg Ile Asn Phe Gln Ser Ser Arg Gly Thr Val Ser Leu  
 50 55 60  
 Gly Leu Lys Arg Asp Asn Leu Tyr Val Val Ala Tyr Leu Ala Met Asp  
 65 70 75 80  
 Asn Thr Asn Val Asn Arg Ala Tyr Tyr Phe Arg Ser Glu Ile Thr Ser  
 85 90 95  
 Ala Glu Ser Thr Ala Leu Phe Pro Glu Ala Thr Thr Ala Asn Gln Lys  
 100 105 110  
 Ala Leu Glu Tyr Thr Glu Asp Tyr Gln Ser Ile Glu Lys Asn Ala Gln  
 115 120 125  
 Ile Thr Gln Gly Asp Gln Ser Arg Lys Glu Leu Gly Leu Gly Ile Asp  
 130 135 140  
 Leu Leu Ser Thr Ser Met Glu Ala Val Asn Lys Lys Ala Arg Val Val  
 145 150 155 160

-155-

Lys Asp Glu Ala Arg Phe Leu Leu Ile Ala Ile Gln Met Thr Ala Glu  
 165 170 175  
~~Ala Ala Arg Phe Arg Tyr Ile Gln Asn Leu Val Ile Lys Asn Phe Pro~~  
 180 185 190  
 Asn Lys Phe Asn Ser Glu Asn Lys Val Ile Gln Phe Glu Val Asn Trp  
 195 200 205  
 Lys Lys Ile Ser Thr Ala Ile Tyr Gly Asp Ala Lys Asn Gly Val Phe  
 210 215 220  
 Asn Lys Asp Tyr Asp Phe Gly Phe Gly Lys Val Arg Gln Val Lys Asp  
 225 230 235 240  
 Leu Gln Met Gly Leu Leu Met Tyr Leu Gly Lys Pro Lys Ser Ser Asn  
 245 250 255  
 Glu Ala Asn

## (2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 813 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGCTAGATA CCGTGTTCATT CTCAACCAAA GGTGCCACTT ATATTACCTA CGTGAATTTT	60
TTGAATGAGC TACGAGTTAA ATTGAAACCC GAAGGTAACA GCCATGGAAT CCCATTGCTG	120
CGCAAAAAT GTGATGATCC TGGAAAGTGT TTCGTTTTGG TAGCGCTTTC AAATGACAAT	180
GGACAGTTGG CGGAAATAGC TATAGATGTT ACAAGTGTTT ATGTGGTGGG CTATCAAGTA	240
AGAAACAGAT CTTACTTCTT TAAAGATGCT CCAGATGCTG CTTACGAAGG CCTCTTCAA	300
AACACAATTA AAACAAGACT TCATTTTGGC GGCAGCTATC CCTCGCTCGA AGGTGAGAAG	360
GCATATAGAG AGACAACAGA CTTGGGCATT GAACCATTAA GGATTGGCAT CAAGAACTT	420
GATGAAAATG CGATAGACAA TTATAAACCA ACGGAGATAG CTAGTTCTCT ATTGTTGTT	480
ATTCAAATGG TGTCTGAAGC AGCTCGATTC ACCTTTATTG AGAACCAAAT TAGAAATAAC	540
TTTCAACAGA GAATTCGCCC GGCGAATAAT ACAATCAGCC TTGAGAATAA ATGGGGTAAA	600
CTCTCGTTCC AGATCCGGAC ATCAGGTGCA AATGGAATGT TTTGGGAGGC AGTTGAATTG	660
GAACGTGCAA ATGGCAAAAA ATACTATGTC ACCGCAGTTG ATCAAGTAAA ACCCAAAATA	720
GCACTCTTGA AGTTCGTGCA TAAAGATCCT AAAACGAGCC TTGCTGCTGA ATTGATAATC	780
CAGAACTATG AGTCATTAGT GGGCTTTGAT TAG	813

-156-

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 846 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

ATGGCGGCAA AGATGGCGAA GAACGTGGAC AAGCCGCTCT TCACCGCGAC GTTCAACGTC      60
CAGGCCAGCT CCGCCGACTA CGCCACCTTC ATCGCCGGCA TCCGCAACAA GCTCCGCAAC      120
CCGGCGCACT TCTCCACAA CCGCCCCGTC CTGCCGCCGG TCGAGCCCAA CGTCCCGCCG      180
AGCAGGTGGT TCCACGTCGT GCTCAAGGCC TCGCCGACCA GCGCCGGGCT CACGCTGGCC      240
ATCCGCGCGG ACAACATCTA CCTGGAGGGG TTCAAGAGCA GCGACGGCAC CTGGTGGGAG      300
CTCACCCCGG GCCTCATCCC CGGCGCCACC TACGTCGGGT TCGGCGGCAC CTACCGCGAC      360
CTCCTCGGCG ACACCGACAA GCTAACCAAC GTCGCTCTCG GCCGACAGCA GCTGGCGGAC      420
GCGGTGACCG CGCTCCACGG GCGCACCAAG GCCGACAAGG CCTCCGGCCC GAAGCAGCAG      480
CAGGCGAGGG AGGCGGTGAC GACGCTGGTC CTCATGGTGA ACGAGGCCAC GCGGTTCCAG      540
ACGGTGTCTG GGTTCGTGGC CGGGTTGCTG CACCCCAAGG CCGTGGAGAA GAAGAGCGGG      600
AAGATCGGCA ATGAGATGAA GGCCCAGGTG AACGGGTGGC AGGACCTGTC CGCGGCGCTG      660
CTGAAGACGG ACGTGAAGCC TCCGCCGGGA AAGTCGCCAG CGAAGTTCGC GCCGATCGAG      720
AAGATGGGCG TGAGGACGGC TGAACAGGCC GCCAACACGC TGGGGATCCT GCTGTTCTGT      780
GAGGTGCCCG GTGGGTTGAC GGTGGCCAAG GCGCTGGAGC TGTTCATGC GAGTGGTGGG      840
AAATAG                                           846

```

## (2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 913 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

CGTCCGAAAA TGGTGAAATG CTTACTACTT TCTTTTAA TTATCGCCAT CTCATTGGT      60
GTTCTACTTG CCAAAGGCGA TGTTAACTTC GATTGTGCGA CTGCCACTGC AAAAACCTAC      120
ACAAAATTTA TCGAAGATTT CAGGGCGACT CTTCCATTTA GCCATAAAGT GTATGATATA      180
CCTCTACTGT ATTCCACTAT TTCCGACTCC AGACGTTTCA TACTCCTCGA TCTTACAAGT      240
TATGCATATG AAACCATCTC GGTGGCCATA GATGTGACGA ACGTTTATGT TGTGGCGTAT      300

```



-157-

CGCACCCGCG ATGTATCCTA CTTTTTTAAA GAATCTCCTC CTGAAGCTTA TAACATCCTA	360
TTCAAAGGTA CGCGGAAAAT TAACTGCCA TATACCGGTA ATTATGAAAA TCTTCAAACCT	420
GCTGCACACA AAATAAGAGA GAATATTGAT CTTGGACTCC CTGCCTTGAG TAGTGCCATT	480
ACCACATTGT TTTATTACAA TGCCCAATCT GCTCCTTCTG CATTGCTTGT ACTAATCCAG	540
ACGACTGCAG AAGCTGCAAG ATTTAAGTAT ATCGAGCGAC ACGTTGCTAA GTATGTTGCC	600
ACTAACTTTA AGCCAAATCT AGCCATCATA AGCTTGAAA ATCAATGGTC TGCTCTCTCC	660
AACAAATCTT TTTGGCGCAG AATCAAGGAG GAAAATTTAG AAATCCTGTC GACCTTATAA	720
AACCTACCGG GGAACGGTTT CAAGTAACCA ATGTTGATTC AGATGTTGTA AAAGGTAATA	780
TCAAACCTCT GCTGAATCC AGAGCTAGCA CTGCTGATGA AAACCTTATC ACAACCATGA	840
CTCTACTTGG GGAATCTGTT GTGAATTGAA AGTTTAATAA TCCACCCATA TCGAAATAAG	900
GCATGTTTCAT GAC	913

## (2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTYAARGAYG CNCCNGAYGC NGCNTAYGAR GG	32
-------------------------------------	----

## (2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TTYTTYATRA TRCANTGNCG NCANCTRGTY CA	32
-------------------------------------	----

## (2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-158-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGNYTNGAYA CNGTNWSNTT YWSNACNAAR GG

32

## (2) INFORMATION-FOR SEQ-ID-NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TGTCTGAACC CGTAACTTGG TAA

23

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CACTCTTCCG TATCTCTCTG T

21

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCAACCCGGG CTAGATACCG TGTCATTCTC AACCAAAGGT GCCACTTATA TTA

53

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTTCATTTTG GCGGCACGTA TCC

23

-159-

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 46 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTCGAGGCTG CAAGCTTACG TGGGATTTTT TTTTTTTTTT TTTTTT

46

## (2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTCGCTGGAA GGTGAGAA

18

## (2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CTCGAGGCTG CAAGCTTACG TGGGA

25

## (2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGATCTCGAG TACTATTTAG GATCTTTATC GACGA

35

-160-

## (2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GTAAGCAGCA TCTGGAGCAT CT

22

## (2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

CATTCAAGAA ATTCACCTAG G

21

## (2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGCCTGGACA CCGTGAGCTT TAG

23

## (2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TCGATTGCGA TCCTAAATAG TACTC

25

-161-

## (2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTTAGGATCG CAATCGACGA ACTTCAAG

28

## (2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GTTCTGTCTGT AAAGATCCTA AATAGTACTC GA

32

## (2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GGATCTTTAC AGACGAACTT CAAGAGT

27

## (2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCTTGTGCTT CGTCGATAAA GATCC

25

-162-

## (2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ATCGACGAAG CACAAGAGTG CTATTTT

27

## (2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GTAAACCAT GCATAGCACT CTTGAAGTTC GT

32

## (2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 32 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

AGTGCTATGC ATGGTTTTAC TTGATCAACT GC

32

## (2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGCACATGTG GTGCCACTTA TATTACCTA

29

-163-

## (2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TAAGTGGCAC CACATGTGCT AAAGCTCAG GTG

33

## (2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 25 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TGACTGTGGA CAGTTGGCGG AAATA

25

## (2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GCCACTGTCC ACAGTCATTT GAAAGCGCTA CC

32

## (2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 36 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GATGATCCTG GAAAGGCTTT CGTTTTGGTA GCGCTT

36

-164-

## (2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 41 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AAGCCTTTC AGGATCATCA GCTTTTGG GCAGCAATGG G

41

## (2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 23 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AAGCCTTTC AGGATCATCA CAT

23

## (2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 18 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GCGACTCTCT ACTGTTTC

18

## (2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 21 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CGTTAGCAAT TTAAGTGA T

21



-165-

## (2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:  
----- (A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AACAGCTATG ACCATG

16

## (2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 29 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TGAACCTCGAG GAAACTACCT ATTTCCCAC

29

## (2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCATTACATC CATGGCGGC

19

## (2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 64 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

GATATCTCGA GTTAACTATT TCCCACCACA CGCATGGAAC AGCTCCAGCG CCTTGCCAC

60

CGTC

64

-166-

## (2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: ~~nucleic acid~~
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TGTCTGTTTC TGGAGGTGCC G

21

## (2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 27 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

CCAAGTGTCT GGAGCTGTTC CATGCCA

27

## (2) INFORMATION FOR SEQ ID NO:51:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

GATGTTAAYT TYGAYTTGTC NACDGCTAC

29

## (2) INFORMATION FOR SEQ ID NO:52:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

ATTGGNAGDG TAGCCCTRAA RTCYTCDAT

29

-167-

## (2) INFORMATION FOR SEQ ID NO:53:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 32 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

GCCACTGCAA AAACCTACAC AAAATTATT GA

32

## (2) INFORMATION FOR SEQ ID NO:54:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

GATGTAACT TCGATTGTC GA

22

## (2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

TCAACTCGAG GTACTCAATT CACAACAGAT TCC

33

## (2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Cys His His His Ala Ser Arg Val Ala Arg Met Ala Ser Asp Glu Phe  
 1 5 10 15

-168-

Pro Ser Met Cys  
20

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

Pro Ser Gly Gln Ala Gly Ala Ala Ala Ser Glu Ser Leu Phe Ile Ser  
1 5 10 15

Asn His Ala Tyr  
20

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 22 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

CAGCCATGGA ATCCATTGC TG

22

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

CACATGTAAA ACAAGACTTC ATTTTGGC

28

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 36 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-169-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

TGAAGTCTTG TTTTAGATGT GTTTTGAAG AGGCCT

36

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ATGCCATATG CAATTATAAA CCAACGGAGA

30

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GGTTTATAAT TGCATATGGC ATTTTCATCA AGTTTCTTG

39

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

CTTCAACAA TGCATTCGCC CGGCGAATAA TAC

33

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

CGGAATGCAT TGTGAAAAGT TATTTCTAAT TTG

33

-170-

## (2) INFORMATION FOR SEQ ID NO:65:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

GTTTTGTGAG GCAGTTGAAT TGGAAC

26

## (2) INFORMATION FOR SEQ ID NO:66:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

TTCAACTGCC TCACAAAACA TTCCATTGTC ACCT

34

## (2) INFORMATION FOR SEQ ID NO:67:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 24 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

AAAAGCTGAT GATCCTGGAA AGTG

24

## (2) INFORMATION FOR SEQ ID NO:68:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

TCCAGGATCA TCAGCTTTT TGCGCAGCAA TGGGA

35

-171-

## (2) INFORMATION FOR SEQ ID NO:69:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 321 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

GACATCCAGA TGACTCAGTC TCCATCTTCC ATGTCTGCAT CTCTGGGAGA CAGAGTCACT	60
ATCACTTGCC GGGCGAGTCA GGACATTAAT AGCTATTTAA GCTGGTTCCA GCAGAAACCA	120
GGGAAATCTC CTAAGACCCCT GATCTATCGT GCAAACAGAT TGGTAGATGG GGTCCCATCA	180
AGGTTTCAGTG GCACTGGATC TGGGACAGAT TATACTCTCA CCATCAGCAG CCTGCAATAT	240
GAAGATTTTG GAATTTATTA TTGTCAACAG TATGATGAGT CTCCGTGGAC GTTCGGTGGA	300
GGCACCAAGC TTGAATCAA A	321

## (2) INFORMATION FOR SEQ ID NO:70:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 354 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

CAGATCCAGT TGGTGAGTC TGGACCTGGC CTGAAGAAGC CTGGAGGGTC CGTCAGAATC	60
TCCTGCGCAG CTTCTGGGTA TACCTTCACA AACTATGGAA TGAAGTGGGT GAAGCAGGCT	120
CCAGGAAAGG GTTTAAGGTG GATGGGCTGG ATAAACACCC AACTGGAGA GCCAACATAT	180
GCTGATGACT TCAAGGACG GTTTACCTTC TCTTTGGACA CGTCTAAGAG CACTGCCTAT	240
TTACAGATCA ACAGCCTCAG AGCCGAGGAC ACGGCTACAT ATTTCTGTAC AAGACGGGGT	300
TACGACTGGT ACTTCGATGT CTGGGGCCAA GGGACCACGG TCACCGTCTC CTCC	354

## (2) INFORMATION FOR SEQ ID NO:71:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 354 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

GAGATCCACT TGGTGAGTC TGGAGGAGGC CTGGTGAAGC CTGGAGGGTC CGTCAGAATC	60
--	----

-172-

TCCTGCGCAG CTTCTGGGTA TACCTTCACA AACTATGGAA TGAAGTGGGT GCGCCAGGCT 120  
 CCAGGAAAGG GTTTAGAGTG GATGGGCTGG ATAAACACCC AACTGGAGA GCCAACATAT 180  
 GCTGATTCTT TCAAGGGACG GTTTACCTTC TCTTTGGACG ATTCTAAGAA CACTGCCTAT 240  
 TTACAGATCA ACAGCCTCAG AGCCGAGGAC ACGGCTGTGT ATTTCTGTAC AAGACGGGGT 300  
 TACGACTGGT ACTTCGATGT CTGGGGCCAA GGGACCACGG TCACCGTCTC CTCC 354

## (2) INFORMATION FOR SEQ ID NO:72:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 321 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

GACATCCAGA TGACTCAGTC TCCATCTTCC CTGCTGCAT CTGTAGGAGA CAGAGTCACT 60  
 ATCACTTGCC GGGCGAGTCA GGACATTAAT AGCTATTTAA GCTGGTTCCA GCAGAAACCA 120  
 GCGAAAGCTC CTAAGACCCT GATCTATCGT GCAAACAGAT TGGAACTCTGG GGTCCCATCA 180  
 AGGTTCACTG GCAGTGGATC TGGGACAGAT TATACTCTCA CCATCAGCAG CCTGCAATAT 240  
 GAAGATTTTG GAATTTATTA TTGTCAACAG TATGATGAGT CTCCGTGGAC GTTCGGTGGA 300  
 GGCACCAAGC TTGAAATCAA A 321

## (2) INFORMATION FOR SEQ ID NO:73:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 70 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

TGTCATCATC ATGCATCGCG AGTTGCCAGA ATGGCATCTG ATGAGTTTCC TTCTATGTGC 60  
 GCAAGTACTC 70

## (2) INFORMATION FOR SEQ ID NO:74:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 78 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA



-173-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

TCGAGAGTAC TTGCGCACAT AGAAGGAAAC TCATCAGATG CCATTCTGGC AACTCGCGAT 60  
GCATGATGAT GACATGCA 78

## (2) INFORMATION FOR SEQ ID NO:75:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TGTTTCGGCCG CATGTCATCA TCATGCATCG 30

## (2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

AGTCATGCCC CGCGC 15

## (2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

TCCCGGCTGT CCTACAGT 18

## (2) INFORMATION FOR SEQ ID NO:78:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

-174-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

TCCAGCCTGT CCAGATGGTG TGTGAGTTTT GTCACAA

37

(2) INFORMATION FOR SEQ ID NO:79:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 76 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CTAACTCGAG AGTACTGTAT GCATGGTTCG AGATGAACAA AGATTCTGAG GCTGCAGCTC

60

CAGCCTGTCC AGATGG

76

(2) INFORMATION FOR SEQ ID NO:80:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

CTAACTCGAG AGTACTGTAT

20

(2) INFORMATION FOR SEQ ID NO:81:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:81:

TCCAGCCTGT CCAGATGGAC ACTCTCCCCT GTTGAA

36

(2) INFORMATION FOR SEQ ID NO:82:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-175-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:82:

GTACAGTGGG AGGTGGAT

18

(2) INFORMATION FOR SEQ ID NO:83:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:83:

CATGCGGCCG ATTAGGATC TTTATCGACG A

31

(2) INFORMATION FOR SEQ ID NO:84:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:84:

AACATCCAGT TGGTGCAGTC TG

22

(2) INFORMATION FOR SEQ ID NO:85:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:85:

GAGGAGACGG TGACCGTGGT

20

(2) INFORMATION FOR SEQ ID NO:86:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 19 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:86:

GACATCAAGA TGACCCAGT

19

-176-

## (2) INFORMATION FOR SEQ ID NO:87:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:87:

GTTTGATTTC AAGCTTGGTG C

21

## (2) INFORMATION FOR SEQ ID NO:88:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 31 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:88:

ACTTCGGCCG CACCATCTGG ACAGGCTGGA G

31

## (2) INFORMATION FOR SEQ ID NO:89:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 723 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:89:

GACATCCAGA TGACTCAGTC TCCATCTTCC CTGTCTGCAT CTGTAGGAGA CAGAGTCACT	60
ATCACTTGCC GGGCGAGTCA GGACATTAAT AGCTATTTAA GCTGGTTCCA GCAGAAACCA	120
GGGAAAGCTC CTAAGACCCT GATCTATCGT GCAAACAGAT TGAATCTGG GGTCCCATCA	180
AGGTTCACTG GCACTGGATC TGGGACAGAT TATACTCTCA CCATCAGCAG CCTGCAATAT	240
GAAGATTTTG GAATTTATTA TTGTCAACAG TATGATGAGT CTCCGTGGAC GTTCGGTGGA	300
GGCACCAAGC TTGAGATGAA AGGTGGCGGT GGATCTGGTG GAGGTGGGTC CGGAGGTGGA	360
GGATCTGAGA TCCAGTTGGT GCAGTCTGGA GGAGGCCTGG TGAAGCCTGG AGGGTCCGTC	420
AGAATCTCCT GCGCAGCTTC TGGGTATACC TTCACAACT ATGGAATGAA CTGGGTGCGC	480
CAGGCTCCAG GAAAGGGTTT AGAGTGGATG GGCTGGATAA ACACCCACAC TGGAGAGCCA	540
ACATATGCTG ATTCTTTCAA GGGACGGTTT ACCTTCTCTT TGGACGATTC TAAGAACACT	600
GCCTATTTAC AGATCAACAG CCTCAGAGCC GAGGACACGG CTGTGTATTT CTGTACAAGA	660

-177-

CGGGGTTACG ACTGGTACTT CGATGTCTGG GGCCAAGGGA CCACGGTCAC CGTCTCCTCA 720  
TGA 723

## (2) INFORMATION FOR SEQ ID NO:90:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 723 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:90:

GAGATCCAGT TGGTGACGTC TGGAGGAGGC CTGGTGAAGC CTGGAGGGTC CGTCAGAATC 60  
 TCCTGCGCAG CTTCTGGGTA TACCTTCACA AACTATGGAA TGAAGTGGGT GCGCCAGGCT 120  
 CCAGGAAAGG GTTTAGAGTG GATGGGCTCG ATAAACACCC AACTGGAGA GCCAACATAT 180  
 GCTGATTCTT TCAAGGGACG GTTTACCTTC TCTTTGGACG ATTCTAAGAA CACTGCCTAT 240  
 TTACAGATCA ACAGCCTCAG AGCCGAGGAC ACGGCTGTGT ATTTCTGTAC AAGACGGGGT 300  
 TACGACTGGT ACTTCGATGT CTGGGGCCAA GGGACCACGG TCACCGTCTC CTCAGGTGGC 360  
 GGTGGATCTG GTGGAGGTGG GTCCGGAGGT GGAGGATCTG ACATCCAGAT GACTCAGTCT 420  
 CCATCTTCCC TGTCTGCATC TGTAGGAGAC AGAGTCACTA TCACTTGCCG GCGGAGTCAG 480  
 GACATTAATA GCTATTTAAG CTGGTTCCAG CAGAAACCAG GGAAAGCTCC TAAGACCCTG 540  
 ATCTATCGTG CAAACAGATT GGAATCTGGG GTCCCATCAA GGTTCAGTGG CAGTGGATCT 600  
 GGGACAGATT ATACTCTCAC CATCAGCAGC CTGCAATATG AAGATTTTGG AATTTATTAT 660  
 TGTCAACAGT ATGATGAGTC TCCGTGGACG TTCGGTGGAG GCACCAAGCT TGAGATGAAA 720  
 TGA 723

## (2) INFORMATION FOR SEQ ID NO:91:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 51 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:91:

CGGACCCACC TCCACCAGAT CCACCGCCAC CTTTCATCTC AAGCTTGGTG C 51

## (2) INFORMATION FOR SEQ ID NO:92:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 19 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

-178-

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:92:

GACATCCAGA TGACTCAGT

19

(2) INFORMATION FOR SEQ ID NO:93:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 49 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:93:

GGTGCAGGTG GGTCCGGAGG TGGAGGATCT GAGATCCAGT TGGTGCAGT

49

(2) INFORMATION FOR SEQ ID NO:94:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:94:

TGTACTCGAG CCCATCATGA GGAGACGGTG ACCGT

35

(2) INFORMATION FOR SEQ ID NO:95:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:95:

GACATCCAGA TGACTCAGT

(2) INFORMATION FOR SEQ ID NO:96:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-179-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:96:

TGTA CTGAG CCCATCATTT CATCTCAAGC TTGGTGC

37

(2) INFORMATION FOR SEQ ID NO:97:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:97:

GAGATCCAGT TGGTGCAGTC TG

22

(2) INFORMATION FOR SEQ ID NO:98:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 49 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:98:

CGGACCCACC TCCACCAGAT CCACCGCCAC CTGAGGAGAC GGTGACCGT

49

(2) INFORMATION FOR SEQ ID NO:99:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 251 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:99:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
1 5 10 15

Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
20 25 30

Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly  
35 40 45

Lys Ala Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
50 55 60

Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
65 70 75 80

Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
85 90 95

-180-

Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
                     100                    105                    110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
                     115                    120                    125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
                     130                    135                    140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
                     145                    150                    155                    160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
                     165                    170                    175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
                     180                    185                    190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
                     195                    200                    205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
                     210                    215                    220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
                     225                    230                    235                    240  
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
                     245                    250

## (2) INFORMATION FOR SEQ ID NO:100:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 251 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:100:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1                    5                    10                    15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
                     20                    25                    30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Ala Asp Asp Pro Gly  
                     35                    40                    45  
 Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
                     50                    55                    60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65                    70                    75                    80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
                     85                    90                    95  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
                     100                    105                    110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
                     115                    120                    125



-181-

Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140

Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160

Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175

Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190

Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205

Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220

Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240

Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:101:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 251 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:101:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15

Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30

Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Ala Asp Asp Pro Gly  
 35 40 45

Lys Ala Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60

Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80

Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95

Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110

Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125

Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140

Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160

Ile	Gln	Met	Val	Ser	Glu	Ala	Ala	Arg	Phe	Thr	Phe	Ile	Glu	Asn	Gln	
				165					170						175	
Ile	Arg	Asn	Asn	Phe	Gln	Gln	Arg	Ile	Arg	Pro	Ala	Asn	Asn	Thr	Ile	
			180					185						190		
Ser	Leu	Glu	Asn	Lys	Trp	Gly	Lys	Leu	Ser	Phe	Gln	Ile	Arg	Thr	Ser	
		195					200					205				
Gly	Ala	Asn	Gly	Met	Phe	Ser	Glu	Ala	Val	Glu	Leu	Glu	Arg	Ala	Asn	
	210					215					220					
Gly	Lys	Lys	Tyr	Tyr	Val	Thr	Ala	Val	Asp	Gln	Val	Lys	Pro	Lys	Ile	
225					230					235					240	
Ala	Leu	Leu	Lys	Phe	Val	Asp	Lys	Asp	Pro	Lys						
				245					250							

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 251 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:102:

Gly 1	Leu	Asp	Thr	Val 5	Ser	Phe	Ser	Thr	Lys 10	Gly	Ala	Thr	Tyr	Ile 15	Thr
Tyr	Val	Asn	Phe 20	Leu	Asn	Glu	Leu	Arg 25	Val	Lys	Leu	Lys	Pro 30	Glu	Gly
Asn	Ser	His 35	Gly	Ile	Pro	Leu	Leu 40	Arg	Lys	Lys	Cys	Asp 45	Asp	Pro	Gly
Lys	Cys 50	Phe	Val	Leu	Val	Ala 55	Leu	Ser	Asn	Asp	Asn 60	Gly	Gln	Leu	Ala
Glu 65	Ile	Ala	Ile	Asp	Val 70	Thr	Ser	Val	Tyr	Val 75	Val	Gly	Tyr	Gln	Val 80
Arg	Asn	Arg	Ser	Tyr 85	Phe	Phe	Lys	Asp	Ala 90	Pro	Asp	Ala	Ala	Tyr 95	Glu
Gly	Leu	Phe	Lys 100	Asn	Thr	Ile	Lys	Thr 105	Arg	Leu	His	Phe	Gly 110	Gly	Ser
Tyr	Pro	Ser 115	Leu	Glu	Gly	Glu	Lys 120	Ala	Tyr	Arg	Glu	Thr 125	Thr	Asp	Leu
Gly	Ile 130	Glu	Pro	Leu	Arg	Ile 135	Gly	Ile	Lys	Lys	Leu 140	Asp	Glu	Asn	Ala
Ile 145	Asp	Asn	Tyr	Lys	Pro 150	Thr	Glu	Ile	Ala	Ser 155	Ser	Leu	Leu	Val	Val 160
Ile	Gln	Met	Val	Ser 165	Glu	Ala	Ala	Arg	Phe 170	Thr	Phe	Ile	Glu	Asn 175	Gln
Ile	Arg	Asn	Asn 180	Phe	Gln	Gln	Arg	Ile 185	Arg	Pro	Ala	Asn	Asn 190	Thr	Ile

-183-

Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205

Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220

Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240

Ala Leu Leu Lys Phe Val Cys Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:103:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 251 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:103:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15

Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30

Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly  
 35 40 45

Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60

Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80

Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95

Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110

Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125

Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140

Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160

Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175

Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190

Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205

Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220

-184-

Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240

Ala Leu Leu Lys Phe Val Asp Cys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:104:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 251 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:104:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly  
 35 40 45  
 Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Cys Ile  
 225 230 235 240  
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
 245 250

-185-

## (2) INFORMATION FOR SEQ ID NO:105:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 251-amino-acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:105:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly  
 35 40 45  
 Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240  
 Ala Leu Leu Cys Phe Val Asp Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:106:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 251 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

-186-

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID-NO:106

Gly Leu Asp Thr Val Ser Phe Ser Thr Cys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly  
 35 40 45  
 Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240  
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
 245 250

(2) INFORMATION FOR SEQ ID NO:107:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 251 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:107:

-187-

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
~~Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly~~  
 20 25 30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Cys Asp Asp Pro Gly  
 35 40 45  
 Lys Cys Phe Val Leu Val Ala Leu Ser Asn Asp Cys Gly Gln Leu Ala  
 50 55 60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240  
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:108:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 251 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:108:

Gly Leu Asp Thr Val Ser Phe Ser Thr Lys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30

Asn	Ser	His	Gly	Ile	Pro	Leu	Leu	Arg	Lys	Lys	Cys	Asp	Asp	Pro	Gly
		35					40					45			
Lys	Cys	Phe	Val	Leu	Val	Ala	Leu	Ser	Asn	Asp	Asn	Gly	Gln	Leu	Ala
	50					55					60				
Glu	Ile	Ala	Ile	Asp	Val	Thr	Ser	Val	Tyr	Val	Val	Gly	Tyr	Gln	Val
65					70					75					80
Arg	Asn	Arg	Ser	Tyr	Phe	Phe	Lys	Asp	Ala	Pro	Asp	Ala	Ala	Tyr	Glu
				85					90					95	
Gly	Leu	Phe	Lys	Asn	Thr	Cys	Lys	Thr	Arg	Leu	His	Phe	Gly	Gly	Ser
			100					105					110		
Tyr	Pro	Ser	Leu	Glu	Gly	Glu	Lys	Ala	Tyr	Arg	Glu	Thr	Thr	Asp	Leu
		115					120					125			
Gly	Ile	Glu	Pro	Leu	Arg	Ile	Gly	Ile	Lys	Lys	Leu	Asp	Glu	Asn	Ala
	130					135					140				
Ile	Asp	Asn	Tyr	Lys	Pro	Thr	Glu	Ile	Ala	Ser	Ser	Leu	Leu	Val	Val
145					150					155					160
Ile	Gln	Met	Val	Ser	Glu	Ala	Ala	Arg	Phe	Thr	Phe	Ile	Glu	Asn	Gln
				165					170					175	
Ile	Arg	Asn	Asn	Phe	Gln	Gln	Arg	Ile	Arg	Pro	Ala	Asn	Asn	Thr	Ile
			180					185					190		
Ser	Leu	Glu	Asn	Lys	Trp	Gly	Lys	Leu	Ser	Phe	Gln	Ile	Arg	Thr	Ser
		195					200					205			
Gly	Ala	Asn	Gly	Met	Phe	Ser	Glu	Ala	Val	Glu	Leu	Glu	Arg	Ala	Asn
	210					215					220				
Gly	Lys	Lys	Tyr	Tyr	Val	Thr	Ala	Val	Asp	Gln	Val	Lys	Pro	Lys	Ile
225					230					235					240
Ala	Leu	Leu	Lys	Phe	Val	Asp	Lys	Asp	Pro	Lys					
				245					250						

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 251 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:109:

Gly	Leu	Asp	Thr	Val	Ser	Phe	Ser	Thr	Lys	Gly	Ala	Thr	Tyr	Ile	Thr
1				5					10					15	
Tyr	Val	Asn	Phe	Leu	Asn	Glu	Leu	Arg	Val	Lys	Leu	Lys	Pro	Glu	Gly
			20					25					30		
Asn	Ser	His	Gly	Ile	Pro	Leu	Leu	Arg	Lys	Lys	Cys	Asp	Asp	Pro	Gly
		35					40					45			
Lys	Cys	Phe	Val	Leu	Val	Ala	Leu	Ser	Asn	Asp	Asn	Gly	Gln	Leu	Ala
	50					55					60				



-189-

Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
~~Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu~~  
~~85 90 95~~  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Cys Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240  
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:110:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 251 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:110:

Gly Leu Asp Thr Val Ser Phe Ser Thr Cys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Ala Asp Asp Pro Gly  
 35 40 45  
 Lys Ala Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95

-190-

Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125  
 Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
 Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240  
 Ala Leu Leu Lys Phe Val Asp Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:111:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 251 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:111:

Gly Leu Asp Thr Val Ser Phe Ser Thr Cys Gly Ala Thr Tyr Ile Thr  
 1 5 10 15  
 Tyr Val Asn Phe Leu Asn Glu Leu Arg Val Lys Leu Lys Pro Glu Gly  
 20 25 30  
 Asn Ser His Gly Ile Pro Leu Leu Arg Lys Lys Ala Asp Asp Pro Gly  
 35 40 45  
 Lys Ala Phe Val Leu Val Ala Leu Ser Asn Asp Asn Gly Gln Leu Ala  
 50 55 60  
 Glu Ile Ala Ile Asp Val Thr Ser Val Tyr Val Val Gly Tyr Gln Val  
 65 70 75 80  
 Arg Asn Arg Ser Tyr Phe Phe Lys Asp Ala Pro Asp Ala Ala Tyr Glu  
 85 90 95  
 Gly Leu Phe Lys Asn Thr Ile Lys Thr Arg Leu His Phe Gly Gly Ser  
 100 105 110  
 Tyr Pro Ser Leu Glu Gly Glu Lys Ala Tyr Arg Glu Thr Thr Asp Leu  
 115 120 125

-191-

Gly Ile Glu Pro Leu Arg Ile Gly Ile Lys Lys Leu Asp Glu Asn Ala  
 130 135 140  
~~Ile Asp Asn Tyr Lys Pro Thr Glu Ile Ala Ser Ser Leu Leu Val Val~~  
 145 150 155 160  
 Ile Gln Met Val Ser Glu Ala Ala Arg Phe Thr Phe Ile Glu Asn Gln  
 165 170 175  
 Ile Arg Asn Asn Phe Gln Gln Arg Ile Arg Pro Ala Asn Asn Thr Ile  
 180 185 190  
 Ser Leu Glu Asn Lys Trp Gly Lys Leu Ser Phe Gln Ile Arg Thr Ser  
 195 200 205  
 Gly Ala Asn Gly Met Phe Ser Glu Ala Val Glu Leu Glu Arg Ala Asn  
 210 215 220  
 Gly Lys Lys Tyr Tyr Val Thr Ala Val Asp Gln Val Lys Pro Lys Ile  
 225 230 235 240  
 Ala Leu Leu Lys Phe Val Cys Lys Asp Pro Lys  
 245 250

## (2) INFORMATION FOR SEQ ID NO:112:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:112:

TGATGCGGCC GACATCTCAA GCTTGGTGC

29

## (2) INFORMATION FOR SEQ ID NO:113:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:113:

TGATGCGGCC GACATCTCAA GCTTGGTGC

29

## (2) INFORMATION FOR SEQ ID NO:114:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 38 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-192-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:114:

TCTAGGTCAC CGTCTCCTCA CCATCTGGAC AGGCTGGA

38

(2) INFORMATION FOR SEQ ID NO:115:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 37 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:115:

TTCGAAGCTT GAGATGAAAC CATCTGGACA GGCTGGA

37

(2) INFORMATION FOR SEQ ID NO:116:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 22 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:116:

GAGATCCAGT TGGTGCAGTC TG

22

(2) INFORMATION FOR SEQ ID NO:117:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 98 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:117:

AGTCGTCGAC ACGATGGACA TGAGGACCCC TGCTCAGTTT CTTGGCATCC TCCTACTCTG

60

GTTTCCAGGT ATCAAATGTG ACATCCAGAT GACTCAGT

98

(2) INFORMATION FOR SEQ ID NO:118:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 80 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-193-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:118:

TCACTTGCCG GCGAATCAG GACATTAATA GCTATTTAAG CTGGTTCCAG CAGAAACCAG 60  
GGAAAGCTCC TAAGACCCT 80

## (2) INFORMATION FOR SEQ ID NO:119:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 80 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:119:

TGACTCGCCC GGCAAGTGAT AGTGACTCTG TCTCCTACAG ATGCAGACAG GGAAGATGGA 60  
GACTGAGTCA TCTGGATGTC 80

## (2) INFORMATION FOR SEQ ID NO:120:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 79 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:120:

GATCCACTGC CACTGAACCT TGATGGGACC CCAGATTCCA ATCTGTTTGC ACGATAGATC 60  
AGGGTCTTAG GAGCTTTCC 79

## (2) INFORMATION FOR SEQ ID NO:121:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 82 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:121:

GGTTCAGTGG CAGTGGATCT GGGACAGATT ATACTCTCAC CATCAGCAGC CTGCAATATG 60  
AAGATTTTGG AATTATTAT TG 82

-194-

## (2) INFORMATION FOR SEQ ID NO:122:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 82 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:122:

GTTTGATTTC AAGCTTGGTG CCTCCACCGA ACGTCCACGG AGACTCATCA TACTGTTGAC 60  
 AATAATAAAT TCCAAAATCT TC

## (2) INFORMATION FOR SEQ ID NO:123:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 107 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:123:

Asp Ile Lys Met Thr Gln Ser Pro Ser Ser Met Tyr Ala Ser Leu Gly  
 1 5 10 15  
 Glu Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Ile Asn Ser Tyr  
 20 25 30  
 Leu Ser Trp Phe His His Lys Pro Gly Lys Ser Pro Lys Thr Leu Ile  
 35 40 45  
 Tyr Arg Ala Asn Arg Leu Val Asp Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Gln Asp Tyr Ser Leu Thr Ile Ser Ser Leu Asp Tyr  
 65 70 75 80  
 Glu Asp Met Gly Ile Tyr Tyr Cys Gln Gln Tyr Asp Glu Ser Pro Trp  
 85 90 95  
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys  
 100 105

## (2) INFORMATION FOR SEQ ID NO:124:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 118 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

-195-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:124:

Gln Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu  
 1 5 10 15  
 Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr  
 20 25 30  
 Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Arg Trp Met  
 35 40 45  
 Gly Trp Ile Asn Thr His Thr Gly Glu Pro Thr Tyr Ala Asp Asp Phe  
 50 55 60  
 Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr  
 65 70 75 80  
 Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Thr Tyr Phe Cys  
 85 90 95  
 Thr Arg Arg Gly Tyr Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr  
 100 105 110  
 Thr Val Thr Val Ser Ser  
 115

## (2) INFORMATION FOR SEQ ID NO:125:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 107 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:125:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Asn Ser Tyr  
 20 25 30  
 Leu Ser Trp Phe Gln Gln Lys Pro Gly Lys Ala Pro Lys Thr Leu Ile  
 35 40 45  
 Tyr Arg Ala Asn Arg Leu Glu Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Asp Tyr Thr Leu Thr Ile Ser Ser Leu Gln Tyr  
 65 70 75 80  
 Glu Asp Phe Gly Ile Tyr Tyr Cys Gln Gln Tyr Asp Glu Ser Pro Trp  
 85 90 95  
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys  
 100 105

-196-

## (2) INFORMATION FOR SEQ ID NO:126:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 118 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:126:

Glu Ile Gln Leu Val Gln Ser Gly Gly Leu Val Lys Pro Gly Gly  
 1 5 10 15  
 Ser Val Arg Ile Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr  
 20 25 30  
 Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Met  
 35 40 45  
 Gly Trp Ile Asn Thr His Thr Gly Glu Pro Thr Tyr Ala Asp Ser Phe  
 50 55 60  
 Lys Gly Arg Phe Thr Phe Ser Leu Asp Asp Ser Lys Asn Thr Ala Tyr  
 65 70 75 80  
 Leu Gln Ile Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Phe Cys  
 85 90 95  
 Thr Arg Arg Gly Tyr Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr  
 100 105 110  
 Thr Val Thr Val Ser Ser  
 115

## (2) INFORMATION FOR SEQ ID NO:127:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 280 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:127:

Ala Ala Lys Met Ala Lys Asn Val Asp Lys Pro Leu Phe Thr Ala Thr  
 1 5 10 15  
 Phe Asn Val Gln Ala Ser Ser Ala Asp Tyr Ala Thr Phe Ile Ala Gly  
 20 25 30  
 Ile Arg Asn Lys Leu Arg Asn Pro Ala His Phe Ser His Asn Arg Pro  
 35 40 45  
 Val Leu Pro Pro Val Glu Pro Asn Val Pro Pro Ser Arg Trp Phe His  
 50 55 60  
 Val Val Leu Lys Ala Ser Pro Thr Ser Ala Gly Leu Thr Leu Ala Ile  
 65 70 75 80



-197-

Arg Ala Asp Asn Ile Tyr Leu Glu Gly Phe Lys Ser Ser Asp Gly Thr  
                     85                    90                    95  
 Trp Trp Glu Leu Thr Pro Gly Leu Ile Pro Gly Ala Thr Tyr Val Gly  
                     100                    105                    110  
 Phe Gly Gly Thr Tyr Arg Asp Leu Leu Gly Asp Thr Asp Lys Leu Thr  
                     115                    120                    125  
 Asn Val Ala Leu Gly Arg Gln Gln Leu Ala Asp Ala Val Thr Ala Leu  
                     130                    135                    140  
 His Gly Arg Thr Lys Ala Asp Lys Ala Ser Gly Pro Lys Gln Gln Gln  
                     145                    150                    155                    160  
 Ala Arg Glu Ala Val Thr Thr Leu Val Leu Met Val Asn Glu Ala Thr  
                     165                    170                    175  
 Arg Phe Gln Thr Val Ser Gly Phe Val Ala Gly Leu Leu His Pro Lys  
                     180                    185                    190  
 Ala Val Glu Lys Lys Ser Gly Lys Ile Gly Asn Glu Met Lys Ala Gln  
                     195                    200                    205  
 Val Asn Gly Trp Gln Asp Leu Ser Ala Ala Leu Leu Lys Thr Asp Val  
                     210                    215                    220  
 Lys Pro Pro Pro Gly Lys Ser Pro Ala Lys Phe Ala Pro Ile Glu Lys  
                     225                    230                    235                    240  
 Met Gly Val Arg Thr Ala Glu Gln Ala Ala Asn Thr Leu Gly Ile Leu  
                     245                    250                    255  
 Leu Phe Val Glu Val Pro Gly Gly Leu Thr Val Ala Lys Ala Leu Glu  
                     260                    265                    270  
 Leu Phe His Ala Cys Gly Gly Lys  
                     275                    280

## (2) INFORMATION FOR SEQ ID NO:128:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:128:

Ala Ala Lys Met Ala Lys Asn Val Asp Lys Pro Leu Phe Thr Ala Thr  
 1                    5                    10                    15  
 Phe Asn Val Gln Ala Ser Ser Ala Asp Tyr Ala Thr Phe Ile Ala Gly  
                     20                    25                    30  
 Ile Arg Asn Lys Leu Arg Asn Pro Ala His Phe Ser His Asn Arg Pro  
                     35                    40                    45  
 Val Leu Pro Pro Val Glu Pro Asn Val Pro Pro Ser Arg Trp Phe His  
                     50                    55                    60  
 Val Val Leu Lys Ala Ser Pro Thr Ser Ala Gly Leu Thr Leu Ala Ile  
                     65                    70                    75                    80

-198-

Arg	Ala	Asp	Asn	Ile 85	Tyr	Leu	Glu	Gly	Phe 90	Lys	Ser	Ser	Asp	Gly 95	Thr
Trp	Trp	Glu	Leu 100	Thr	Pro	Gly	Leu	Ile 105	Pro	Gly	Ala	Thr	Tyr 110	Val	Gly
Phe	Gly	Gly 115	Thr	Tyr	Arg	Asp	Leu 120	Leu	Gly	Asp	Thr	Asp 125	Lys	Leu	Thr
Asn	Val 130	Ala	Leu	Gly	Arg	Gln 135	Gln	Leu	Ala	Asp	Ala 140	Val	Thr	Ala	Leu
His 145	Gly	Arg	Thr	Lys 150	Ala	Asp	Lys	Ala	Ser	Gly 155	Pro	Lys	Gln	Gln	Gln 160
Ala	Arg	Glu	Ala	Val 165	Thr	Thr	Leu	Val	Leu 170	Met	Val	Asn	Glu	Ala	Thr 175
Arg	Phe	Gln	Thr 180	Val	Ser	Gly	Phe	Val 185	Ala	Gly	Leu	Leu	His 190	Pro	Lys
Ala	Val	Glu 195	Lys	Lys	Ser	Gly	Lys 200	Ile	Gly	Asn	Glu 205	Met	Lys	Ala	Gln
Val	Asn 210	Gly	Trp	Gln	Asp	Leu 215	Ser	Ala	Ala	Leu	Leu 220	Lys	Thr	Asp	Val
Lys 225	Pro	Pro	Pro	Gly	Lys 230	Ser	Pro	Ala	Lys	Phe 235	Ala	Pro	Ile	Glu	Lys 240
Met	Gly	Val	Arg	Thr 245	Ala	Glu	Gln	Ala	Ala 250	Asn	Thr	Leu	Gly	Ile 255	Leu
Leu	Phe	Val	Glu 260	Val	Pro	Gly	Gly	Leu 265	Thr	Val	Ala	Lys	Cys 270	Leu	Glu
Leu	Phe	His 275	Ala	Ser	Gly	Gly	Lys 280								

(2) INFORMATION FOR SEQ ID NO:129:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 280 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ. ID NO:129:

Ala	Ala	Lys	Met	Ala	Lys	Asn	Val	Asp	Lys	Pro	Leu	Phe	Thr	Ala	Thr	
1				5					10					15		
Phe	Asn	Val	Gln	Ala	Ser	Ser	Ala	Asp	Tyr	Ala	Thr	Phe	Ile	Ala	Gly	
			20					25					30			
Ile	Arg	Asn	Lys	Leu	Arg	Asn	Pro	Ala	His	Phe	Ser	His	Asn	Arg	Pro	
		35					40					45				
Val	Leu	Pro	Pro	Val	Glu	Pro	Asn	Val	Pro	Pro	Ser	Arg	Trp	Phe	His	
	50					55					60					
Val	Val	Leu	Lys	Ala	Ser	Pro	Thr	Ser	Ala	Gly	Leu	Thr	Leu	Ala	Ile	
65					70					75					80	

-199-

Arg Ala Asp Asn Ile Tyr Leu Glu Gly Phe Lys Ser Ser Asp Gly Thr  
                             85                            90                            95  
 Trp-Trp-Glu-Leu-Thr Pro Gly Leu Ile Pro Gly Ala Thr Tyr Val Gly  
                             100                            105                            110  
 Phe Gly Gly Thr Tyr Arg Asp Leu Leu Gly Asp Thr Asp Lys Leu Thr  
                             115                            120                            125  
 Asn Val Ala Leu Gly Arg Gln Gln Leu Ala Asp Ala Val Thr Ala Leu  
                             130                            135                            140  
 His Gly Arg Thr Lys Ala Asp Lys Ala Ser Gly Pro Lys Gln Gln Gln  
                             145                            150                            155                            160  
 Ala Arg Glu Ala Val Thr Thr Leu Val Leu Met Val Asn Glu Ala Thr  
                             165                            170                            175  
 Arg Phe Gln Thr Val Ser Gly Phe Val Ala Gly Leu Leu His Pro Lys  
                             180                            185                            190  
 Ala Val Glu Lys Lys Ser Gly Lys Ile Gly Asn Glu Met Lys Ala Gln  
                             195                            200                            205  
 Val Asn Gly Trp Gln Asp Leu Ser Ala Ala Leu Leu Lys Thr Asp Val  
                             210                            215                            220  
 Lys Pro Pro Pro Gly Lys Ser Pro Ala Lys Phe Ala Pro Ile Glu Lys  
                             225                            230                            235                            240  
 Met Gly Val Arg Thr Ala Glu Gln Ala Ala Asn Thr Leu Gly Ile Cys  
                             245                            250                            255  
 Leu Phe Val Glu Val Pro Gly Gly Leu Thr Val Ala Lys Ala Leu Glu  
                             260                            265                            270  
 Leu Phe His Ala Ser Gly Gly Lys  
                             275                            280

## (2) INFORMATION FOR SEQ ID NO:130:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 7 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:130:

Ser Cys Asp Lys Thr His Thr  
 1                            5

## (2) INFORMATION FOR SEQ ID NO:131:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 85 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

-200-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:131:

TGTCGACATC ATGGCTTGGG TGTGGACCTT GCTATTCCTG ATGGCAGCT GCCCAAAGTG 60  
CCCAAGCAGAGATC CAGTTGGTGCA G 82

## (2) INFORMATION FOR SEQ ID NO:132:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 86 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:132:

AAGGTATACC CAGAAGCTGC GCAGGAGATT CTGACGGACC CTCCAGGCTT CACCAGGCCT 60  
CCTCCAGACT GCACCAACTG GATCTC 86

## (2) INFORMATION FOR SEQ ID NO:133:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:133:

GCAGCTTCTG GGTATACCTT CACAACTAT GGAATGAACT GGGTCCGCCA GGCTCCAGGA 60  
AAGAATTTAG AGTGGATGGG CTGG 84

## (2) INFORMATION FOR SEQ ID NO:134:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 85 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:134:

AAAGAGAAGG TAAACCGTCC CTTGAAAGAA TCAGCATATG TTGGCTCTCC AGTGTGGGTG 60  
TTTATCCAGC CCATCCACTC TAAAC 85

-201-

## (2) INFORMATION FOR SEQ ID NO:135:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 87 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:135:

GACGGTTTAC CTTCTCTTTG GACGATTCTA AGAACACTGC CTATTACAG ATCAACAGCC 60  
TCAGAGCCGA GGACACGGCT GTGTATT 87

## (2) INFORMATION FOR SEQ ID NO:136:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 92 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:136:

GAGGAGACGG TGACCGTGGT CCCTTGGCCC CAGACATCGA AGTACCAGTC GTAACCCCGT 60  
CTTGACAGA AATACACAGC CGTGTCTCTG GC 92

## (2) INFORMATION FOR SEQ ID NO:137:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 84 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:137:

GCAGCTTCTG GGTATACCTT CACAACTAT GGAATGAACT GGGTGAAGCA GGCTCCAGGA 60  
AAGGGTTTAA GGTGGATGGG CTGG 84

## (2) INFORMATION FOR SEQ ID NO:138:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 85 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

-202-

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:138:

AAAGAGAAGG TAAACCGTCC CTTGAAGTCA TCAGCATATG TTGGCTCTCC AGTGTGGGTG 60  
TTTATCCAGC CCATCCACCT TAAAC 85

## (2) INFORMATION FOR SEQ ID NO:139:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 84 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:139:

GACGGTTTAC CTTCTCTTTG GACACGTCTA AGTGCACTGC CTATTTACAG ATCAACAGCC 60  
TCAGAGCCGA GGACACGGCT ACAT 84

## (2) INFORMATION FOR SEQ ID NO:140:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 91 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:140:

AGGAGACGGT GACCGTGGTC CCTTGGCCCC AGACATCGAA GTACCAGTCG TAACCCCGTC 60  
TTGTACAGAA ATATGTAGCC GTGTCCTCGG C 91

## (2) INFORMATION FOR SEQ ID NO:141:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:141:

Lys Pro Ala Lys Phe Phe Arg Leu  
1 5

## (2) INFORMATION FOR SEQ ID NO:142:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 8 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

-203-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:142:

Lys Pro Ala Lys Phe Leu Arg Leu

1-----5-----

(2) INFORMATION FOR SEQ ID NO:143:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:143:

GGCCGCAAAG CCGGCTAAGT TCTTTCGTCT GAGT

34

(2) INFORMATION FOR SEQ ID NO:144:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:144:

ACTCAGACGK AAGAACTTAG CCGGCTTTGC

30

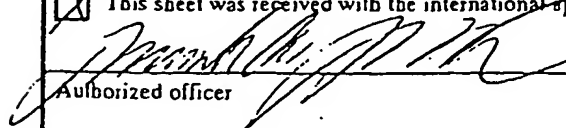
## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page _____, line _____ See attached sheet	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input checked="" type="checkbox"/>	
Name of depositary institution American Type Culture Collection	
Address of depositary institution (including postal code and country) 12301 Parklawn Drive Rockville, Maryland 20852 United States of America	
Date of deposit See attached sheet	Accession Number See attached sheet
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
<p>"In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 23(4) EPC)."</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
EPO	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only

☒ This sheet was received with the international application

  
Authorized officer

For International Bureau use only

☐ This sheet was received by the International Bureau on:

Authorized officer



<u>ATCC Accession Number</u>	<u>Date Deposited</u>	<u>Page No.</u>
68721	October 2, 1991	4
68722	October 2, 1991	9
69008	June 9, 1992	12
69009	June 9, 1992	18
69101	October 27, 1992	18
69102	October 27, 1992	18
69103	October 27, 1992	18
69104	October 27, 1992	18

## CLAIMS

1. A fusion protein comprising gelonin fused to the amino or carboxy terminal end of an antigen-binding portion of he3 antibody.

2. The fusion protein of claim 1 wherein said fusion protein lacks a cleavable peptide segment linking said gelonin and said antibody portion.

3. The fusion protein of claim 1 which is  $V_L V_B$ -Gel.

4. The fusion protein of claim 1 which is  $V_B V_L$ -Gel.

5. The fusion protein of claim 1 which is Gel- $V_B V_L$ .

6. The fusion protein of claim 1 which is Gel- $V_L V_B$ .

7. The fusion protein of claim 1 which is Gel:: $\kappa$ ,Fd.

8. The fusion protein of claim 1 which is Gel:: $\text{Fd}, \kappa$ .

9. A fusion protein comprising gelonin fused to the amino or carboxy terminal end of an antigen-binding portion of an antibody, said fusion protein further comprising the peptide segment CCL between said gelonin and said antibody portion.

10. A fusion protein comprising gelonin fused to the amino or carboxy terminal end of an antigen-binding portion of an antibody, said fusion protein further comprising the peptide segment CCF between said gelonin and said antibody portion.

11. The fusion protein (Gel::RMA::kappa,Fd')<sub>2</sub>.

12. The fusion protein (Gel::RMA::Fd', kappa)<sub>2</sub>.

13. The fusion protein Gelonin::RMA::kappa,Fd.

14. The fusion protein Gelonin::RMA::Fd,kappa.

15. The fusion protein Gelonin::SLT::kappa,Fd.

16. The fusion protein Gelonin::SLT::Fd,kappa.

17. The fusion protein V<sub>H</sub>V<sub>L</sub>::SLT::Gelonin.

18. The fusion protein V<sub>L</sub>V<sub>H</sub>::SLT::Gelonin.

19. The fusion protein V<sub>H</sub>V<sub>L</sub>::RMA::Gelonin.

20. The fusion protein V<sub>L</sub>V<sub>H</sub>::RMA::Gelonin.

21. A polynucleotide encoding the fusion protein of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20.

22. A host cell transformed or transfected with the fusion protein of claim 21.

1 / 14

RTA	IFPKQYPIINFITAGATVDSYTNFIRAVRGRLLTGADVRHEIPVLPNRVG	50
GELONIN	GLD-----TVSESTKGATYITYVNFNLNLRVKLKPEGN-SHGIPLLRKKCD	45
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGVRAGNSAYFFHPDNQED	100
GELONIN	DP-GKCFVLVALSNDNGQLAEIAIDVTSVYVVGQVRNRSYFF---KDA	90
RTA	AEAITHLFTDVQNRRTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
GELONIN	PDAAYEGLFKNTIKTRLHFSGSYPSEKAYRETTDLGIEPLRIGIKK	139
RTA	LYYSTGGTQPLTARSFIICIMISEAARFQYIEGEMRTRIRYNRRSAP	200
GELONIN	LDENAIQNYKPTETIASLLVVIQMVSEAAARFTFIENQIRNN--FQQRIRP	187
RTA	DPSVITLENSWGRSLTAIQESN-QGAFASPIQLORRNGSKFSVDVSILI	249
GELONIN	ANNTISLENKWGKLSFOIRTSGANGHFSEAVELERANGKKYYVVTAVDQVK	237
RTA	PIIALMVYRCAPPSSQF	267
GELONIN	PKIALLKFDVKDPK	251

FIG. 1

2 / 14

RTA	I-----FPKQYPIINFTTAGATVQSYTNFIRAVRGRLLTTGADVREIPV	44
BRIP	AAKMAKNVDKPLFTATFNQASSAD-YATFIAGIRNKLRNPAHFSHNRPV	49
RTA	LPN-RVGLPINQRFILVELSNHAELSVTLALDVTNAYVVGYRAGNSAYFF	93
BRIP	LPPVEPNVPPSRWFHVVLKASPTSAGLTLAIRADNIYLEGFKSSDGTWWE	99
RTA	HPDNQEDAEATHLFTDVQNRYTFAFGGNYDRLEQLAGNLRENIELGNP	143
BRIP	LTPGLIPGATYV-----GFGGTYRDLLGDTDKL-TNVALGRQQ	136
RTA	LEEASALY-----YYSTGGTOLPTLARSFIICIONISEAARFQ-----YIE	185
BRIP	LADAVTALHGRTKADKASGPKQQQAREAVTTLLVMVNEATRFQTVSGFVA	186
RTA	GEMRTRIRYNRRSAPDPSVITLENSWGRSLTAIQESNQGFASPIQLQRR	235
BRIP	GLLHPKAVEKKSGKIGNEMKAQVNGWQDLSAALLKTDVKPPPGKSPAKFA	236
RTA	NGSKFSVYDVSILIPIIALHVYRCAP-----PPSSQF	267
BRIP	PIEKHGVRTAEQAANTLGILLFVEVPGGLTVAKALELFHASGGK	280

FIG. 2

3 / 14

RTA	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
MOM011	D-----VNFDLSTATAKTYTKFIEDFRATLPFSHKV-YDIPLLYSTIS	42
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGYRAGNSAYFFHPDNQED	100
MOM011	--DSRRFILLDLTSAYETISVAIDVTNVVVVAYRTRDVSYFF---KESP	87
RTA	AEAITHLFTDVQNRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
MOM011	PEAYNILFKGTR-KITLPTYGNYENLQTAAHKIRENIDLGLPALSSAITT	136
RTA	LYYSTGGTOLPTILARSFIIICIONISEAARFQYIEGEMRTRIRYNRRSAP	200
MOM011	LFYYNQSA-----PSALLVLIQTTAEAAARFKYIERHVAKYVATNFK--P	179
RTA	DPSVITLENSWGRNSTAI--OESNQGAFAPIQLQRRNGSKFSVYDVS--	246
MOM011	NLAIISLENQWSALS*QIFLAQHQGGKFRNPVDLIKPTGERFQVTNVDSD	229
RTA	ILIPIIALMVYRCAPPPSSQF	267
MOM011	VVKGNIKLLNSRASTADENFITTMTLLGESVNN	263

FIG. 3

4 / 14

RTA	IFPKOYPIINFITAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
LUFFIN	D-----VRFSLSGSSSTSYSKFIGDLRKALPSNGTVVNLTIILLSSASG	43
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGYRAGNSAYFFHPDNQED	100
LUFFIN	---ASRYTLMTLSNYDYGKAITVAVDVSQLYIMGYLVNSTSYFF---NESD	87
RTA	AEAITHLFTDVONRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
LUFFIN	AKLASQYVFKGSTIVTLPYSGNYEKLQTAAGKIREKIPLGFPAldsALT	137
RTA	LYYSTGGTQPLTARSFIICIONISEAARFQYIEGEMRTRIRYNRRSAP	200
LUFFIN	IFHYDSTAA-----AAFLVLQTTAEASRFKYIEGOIERI--SKNQVP	180
RTA	DPSVITLENS-WGRLSTAIQ--ESNQAFASPIQLQRRNGSKFSVYDVS	247
LUFFIN	SLATISLENSLWSLSKQIQLAQTNGTFTKTPVVITDDKQQRVEITNVTS	230
RTA	LPIIIALMVYRCAPPSSQF	267
LUFFIN	KVVTKNIQLLLNKQNV	248

FIG. 4

5 / 14

RTA	IFPKQYPIINFTTAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
TRICHO	D-----VSFRLSGATSSSYGVFISNLRKALPNERKL-YDIPLL--RSS	40
RTA	LPINORFILVELSNHAELSVTLALDVTNAYVVGVRAGNSAYFFHPDNQED	100
TRICHO	LPGSORYALIHLTNYADETISVAIDVTNVYINGYRAGDTSYFF--NEASA	88
RTA	AEAITHLFTDVQNRYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
TRICHO	TEAAKYVFKDAMRKVTLPSYGNRYERLQTAAGRIRENIPLGLPALDSAITT	138
RTA	LYYYSTGGTQLPTLARSFIICIQMISEAARFQYIEGEMRTRIRYNRRSAP	200
TRICHO	LFYYNANSA-----ASALMVLIOSTSEAAARYKFIEQQIGSRV--DKTFLP	181
RTA	DPSVITLENSWGRSLTAIQ--ESNQGAFASPIQLORRNGSKFSVYDV--S	246
TRICHO	SLAIIISLENSWSALSQIQIASTNNGQFESPVLINAQNRVTITNV DAG	231
RTA	ILIPIIALMVYRCAPPSSQF	267
TRICHO	VVTSNIALLLNRNNMA	247

FIG. 5



RTA	IFPKQYPIINFITAGATVQSYTNFIRAVRGRLLTGADVRHEIPVLPNRVG	50
MOMOI	D-----VSFRLSGADPRSYGMFIKDLRNALPFREKVYNIPLLPSVSG	43
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGVRAGNSAYFFHDPDQED	100
MOMOI	---AGRYLLMHLFNYDGKTIITVAVDVTNVYINGYLADTTSYFFNEPAAEL	90
RTA	AEAITHLFTDVQNRNYTFAFGGNYDRLEQLAGNLRENIELGNGLPEEAISA	150
MOMOI	ASQ--YVFRDARRKITLPYSGNYERLQIAAGKPREKIPIGLPALDSAIST	138
RTA	LYYSTGGTQLPTLARSFIICIQMISEARFOYIEGEMRTRIRYNRRSAP	200
MOMOI	LLHYDSTAA-----AGALLVLIQTTAEAAARFKYIEQQIQERA--YRDEVP	181
RTA	DPSVITLENSWGRRLSTAIQ--ESNQGAFAPIQLORRNGSKFSVYDVSIL	248
MOMOI	SLATISLENSWSGLSKQIQLAQGNNGIFRTPIVLVDNKGNRVQITNVTSK	231
RTA	IPIIALHVY-----RCAPPPSSQF	267
MOMOI	VVTSNIQLLLNTRNIAEGDNGDVSTTHGFSST	263

FIG. 6

7 / 14

RTA	IFPKQYPIINF	TAGATVQSYTNF	IRAVRGR	LTTGADVR	HEIPVLP	NRVG	50
MAP	A-PTLETIAS	LDLNNPT--	TYLSFITN	IRTKVADKTE	-----	QCTIQKIS	42
RTA	LPINORFIL	VELSNHAELS	SVTLALD	VTHAYV	VGYRA--	GNSAYFFHPDN	97
MAP	KTFTQRY	SYIDLIV	SSSTOKIT	LAI	DMA	DLYVLGYSDIAN	92
RTA	QEDAE	AITHLFTD	VQNR	YTFAGG	NYDRLEQLAG	NLENIELGN	147
MAP	TEAVANN	FFPGATG	TNR	IKLTFTG	SYGDLEK-NGGLR	KDNPLGIFRL	141
RTA	ISALYY	YSTGGTQL	PTLARS	FIIC	IONI	SEAA	197
MAP	IVNIY	---	GKAGD	VKKQAK	FFLLAION	VSEAA	186
RTA	SAPDPS	VITLENS	WGR	LSTAIQ	ESNQ	GAFASPIQLOR	247
MAP	VTVDEY	HTALENN	WAKL	STAVY	NSKPS	TTTATKCOL	236
RTA	LIP	IIALH	VYR	CA	PP	SSQF	267
MAP	VEE	IKLVH	GL	LK	SS		250

FIG. 7

RTA	IFPKQYPIINFITTAGATVQSYTNFIRAVRGRLLTTGADVRRHEIPVLPNRVG	50
PAPS	I-----NTITFDAGNATINKYATFHESLRNEAKDPSLKCYGIPHLPTNS	45
RTA	LPINQRFILVELSNHAELSVTLALDVTNAYVVGYRAGNSA-----YFFHP	95
PAPS	---TIKYLVLVKLOGASLKTITILMLRRNNLYVMGYSDPYDNKCRYHIFNDI	92
RTA	DNOEDAEATHLFTDVQNRYT--FAFGGNYDRLEQLAG-NLRENIELGNG	142
PAPS	KGTEYSDVENTLCPSSNPRVAKPINYNGLYPTLEKKAGVTSRNEVQLGIG	142
RTA	PLEEAI S A L Y Y S T G G T Q L P T L A R S F I I C I Q M I S E A A R F Q Y I E G E M R T R I	192
PAPS	ILSSDIGKI--SGQGSFTEKIEAKFLLVAIQMVSEAAARFKYIENQVKTN-	189
RTA	RYNRRSAPDPSVITLNSWGRSLTAIQESNQGAFA S P I O L O R R N G S K F S V	242
PAPS	-FNRDFSPNDKVLDLEENWGKISTAIHNSKNGALPKPLELKNADGTKWIV	238
RTA	YDVSILIPIIIALMVYRCAPPSSQF	267
PAPS	LRVDEIKPDVGLLNY--VNGTCQAT	261

FIG. 8

RTA	IFPKQYPIINFITAGATVQSYTNFIRAVRGRLTTGADVRHEIPVLPNRVG	50
SAP6	V-----TSITLDLVNPTAQYSSFVKIRNNVKDPNLKYGGIDI--AVIG	43
RTA	LPINORFILVELSNHAELSVTLALDVTNAYVVVGYRAGNS-----AYFFHP	95
SAP6	PPSKEKFLRINFQSSRG-TVSLGLKRDNLVYVAYLAMDNTNVNRAYYFRS	92
RTA	DNOEDAEATHLFTDVQNRYTFAFGGNYDRLEQ-----LAGNLRNIELG	140
SAP6	EITSAESTALFPEATTAHOKALEYTEDYQSIENAKNAITQGDQSRKELGLG	142
RTA	NGPLEEASALYYSTGGTQPLTLARSFIICIQMISEAARFQYIEGEMRT	190
SAP6	IDLLSTSHEAV---NKKARVVKDEARFLLIAIQHTAEAAFRFYIQNLVIK	189
RTA	RIRYNRRSAPDPSVITLENSWGRLSTAI-QESNQGAFAPIQLORRNGSK	239
SAP6	N--FPNKFENSENKVIOFEVNWKKISTAIYGDAKNGVFNKDYDFGFGKVRQ	237
RTA	FSVYDVSILIPIIALMVYRCAPPPSSQF	267
SAP6	VKDLOMGLL-----MYLGKPKSSNEAH	259

FIG. 9

10 / 14

pos		10	20	30	40	50
bind	+++++	o+++++o+++++	-----	-----	oo++++o++++	-----
bury	+++++	o+o+-----	-----	-----	o++++o-----	-----
mod	.....	.....	.....	.....	.....	.....
hK1	DIQMTQS	PSSLSASVGDRVTITCrASQx	Is	xyLxWYQQKPGkAPkLIIY		aAS
hK3	EIVLTQS	PgTSLSPGERATLSCRASQS	vs	ssyLAWYQQKPGQAPRLIIY		gaS
hK2	DIVMTQS	PLSLPVTGPASISCRSSQS	ll	nnYLhWYLCkPGQSPQLIIY		igS
hL1	ASVLTQP	PS aSgtPGQrVtISCsGssS	iG	xxVxWYQqIPGtAPkLIIY		n n
hL2	ASALTQP	as VSGSPGQSiTISCtGtss	Vg	ynxVSWyQqHPGkAPK LIY		dv
hL3	SYeLTQP	PS vSVsPGQTA ITCsGdx	lx	xxvVxWYQQkPgQaPviVIY		d
hL6	nfmltqp	ns vsespgktvtisctxsxg	la	sxyvqwyqqrpgsapttviy		edn
hK4	divmtqs	pdsLavslgeratinckssqs	vl	knylawyqqkpgqppklliy		was
hL4	seltqp	ps vsvapgqt ritcsGdx	lg	xydaxwyqqkpgqapilviy		grn
hL5	saltqp	ps asgspgqsvtisctgtss	vg	xxvVswyqqh g apk l		ev

pos		60	70	80	90	100
bind	--o++o++o++o++o++	-----	-----	-----	o-----	o++++o-----
bury	++o++o++o++o++o++	-----	-----	-----	-----	-----
mod	.....	.....	.....	.....	.....	.....
hK1	xLxSGVPSRFsGSGSGTx	FTLTISsLQPeDfATYYCqgyxxxP	xt	FGqGTkv eik		
hK3	sRATGIPdRFSGSgSGTD	FTLTISrLEPGDFAVYYCQqYgssP	xt	FGqGTkv EIK		
hK2	nRASGVpDRFSGSGSGTD	FTLkISRVEaEDVGvYYCMQaIqXP	xt	FGqGTkv EIK		
hL1	RPSGVpDRFSGSKSGTS	AsLaIsGLQseDeaDYyCacWDsSLd	pV	FGGGTk TVLg		
hL2	RPSGv RFSGSKSGnT	ASLTISGtQaeDEAdYYCsSYxgxxx	xV	FGgGtktVLg		
hL3	RPSgIPQRFSGS St T	ATLTISGvqa DEAdYYCqxxDxxx	vv	FGGGTkLTVlg		
hL6	rpsgvpdrfsGsgss ns	asltisglkteadeadyycqsyosxx	wv	fgggtkltvlg		
hK4	resgvpdrfsGsgsgtd	ftltissslqaqdvavyyccqyyystp	xt	fgqgtkx sik		
hL4	rpsgipdrfsGsgssght	asltirgaqaeadeadyycnsrdssgk	vl	fgggtkltvlg		
hL5	rpsgvpdrfsGsgks nt	asltvsgl aeadeadyycssyxxxxx	fv	fg gtx tvl		

FIG. 10A

[illegible]

**FIG. 10B**

## SH65K-1

AGT CGT CGA CAC GAT GGA CAT GAG GAC CCC TGC TCA GTT TCT TGG  
CAT CCT CCT ACT CTG GTT TCC AGG TAT CAA ATG TGA CAT CCA GAT  
GAC TCA GT

## HUH-K1

TGA CTC GCC CGG CAA GTG ATA GTG ACT CTG TCT CCC AGA CAT GCA  
GAC ATG GAA GAT GAG GAC TGA GTC ATC TGG ATG TC

## HUH-K2

TCA CTT GCC GGG CGA GTC AGG ACA TTA ATA GCT ATT TAA GCT GGT  
TCC AGC AGA AAC CAG GGA AAT CTC CTA AGA CCC T

## HUH-K3

GAT CCA CTG CCA CTG AAC CTT GAT GGG ACC CCA TCT ACC AAT CTG  
TTT GCA CGA TAG ATC AGG GTC TTA GGA GAT TTC C

## HUH-K4

GGT TCA GTG GCA GTG GAT CTG GGA CAG ATT ATA CTC TCA CCA TCA  
GCA GCC TGC AAT ATG AAG ATT TTG GAA TTT ATT ATT G

## HUH-K5

GTT TGA TTT CAA GCT TGG TGC CTC CAC CGA ACG TCC ACG GAG ACT  
CAT CAT ACT GTT GAC AAT AAT AAA TTC CAA AAT CTT C

## HUH-G1

TGT CGA CAT CAT GGC TTG GGT GTG GAC CTT GCT ATT CCT GAT GGC  
AGC TGC CCA AAG TGC CCA AGC ACA GAT CCA GTT GGT GCA G

## HUH-G2

AAG GTA TAC CCA GAA GCT GCG CAG GAG ATT CTG ACG GAC  
CCT CCA GGC TTC TTC AGG CCA GGT CCA GAC TGC ACC AAC TGG ATC T

## HUH-G3

GCA GCT TCT GGG TAT ACC TTC ACA AAC TAT GGA ATG AAC TGG GTG  
AAG CAG GCT CCA GGA AAG GGT TTA AGG TGG ATG GGC TGG

## HUH-G4

AAA GAG AAG GTA AAC CGT CCC TTG AAG TC A TCA GCA TAT GTT GGC  
TCT CCA GTG TGG GTG TTT ATC CAG CCC ATC CAC CTT AAA C

## HUH-G5

GAC GGT TTA CCT TCT CTT TGG ACA CGT CTA AGT GCA CTG CCT ATT  
TAC AGA TCA ACA GCCTCA GAG CCG AGG ACA CGG CTA CAT

## HUH-G6

AGG AGA CCG TGA CCG TGG TCC CTT GGC CCC AGA CAT CGA AGT ACC  
AGT CGT AAC CCC GTC TTG TAC AGA AAT ATG TAG CCG TGT CCT CGG C

## H65G-23

ACT AGT GTC GAC ATC ATG GCT TGG GT

## H65-G2

GAG GAG ACG GTG ACC GTG GT

## H65K-23

AGT CGT CGA CAC GAT GGA CAT GAG GAC

## JK1-HindIII

GTT TGA TTT CAA GCT TGG TGC

**FIG. 11**

13 / 14

pos		10	20	30	40	50
EU	DIQMTQS	PSTLSASVGDRVTITCRASQS	IN	TWLAWYQQKPGKAPKLLMY	KAS	
hK1	DIQMTQS	PSSLSASVGDRVTITC:ASQx	IS	xyLxWYQQKPGkAPkILlY	aAS	
TAC	QIVLTQS	PAIMSASPGKVTITCSASSS	IS	YMHWFQOKPGTSPKLWIY	TTS	
bind	+++++	o+++++o+++++	---	---ooo++++o++++	---	
bury	+++++	o++o+-----	++	+++---o++++o+++	+++	
mod	.	.	.	.	.	.
M/H	H HM	HHM M HH	h M	M MM M	hm M	MM
prop	DIQMTQS	PSSMSASPGDRVTITCRASSS	IS	YMHWFQOKPGKSPKLWIY	TTS	
Que	DIQMTQS	PSTLSASVGDRVTITCSASSS	IS	YMHWFQOKPGKAPKLLIY	TTS	

pos	60	70	80	90	100
EU	SLESGVPSRFISGSGS	TE	FTLTISSLQPDDEFATYYCQQYNSDS	KM	FGQGTKV EVK
hK1	xLxSGVPSRFsGSGSGTx		FTLTISsLQPeDfATYYCqqyxxxP	xt	FGqGtKv eik
TAC	NLASGVPARFSGSGSGTS		YSLTISRMEAEDAATYYCHQRSTYP	LT	FGSGTKL ELK
bind	---oo++o+++++-----		-----o-----		-o o+++++ +++
bury	++o+++o+o+---+o+o++		-----+-----		--- +----- +++
mod	.	.	.	.	.
M/H	M M	H m	MH hMHm h	M MMM	M h M m
prop	NLASGVPSRFSGSGSGTS		YTLTISSMQAEDFATYYCHQRSTYP	LT	FGQGTKL ELK
Que	NLASGVPARFSGSGSGTE		FTLTISSLQPDDEFATYYCHQRSTYP	LT	FGQGTKV EVK

FIG. 12A



14 / 14

```

pos      10      20      30      40      50
EU      QVQLVQSGAE VKKPGSSVKVSKASGGTFS RSAIIWVRQAPGQGLEWMGGIVPMFGPP
hH1     QVqLvqSGaE VkkPGxSvxxvSCKxSGyyFx xyxixWvRQaPGxGLEWvGxixpxxgx
TAC     QVQLQQSGAE LAKPGASVKMSCKASGYTFT SYRMHWVKQRPQGQLEWIGYINPSTGYT
bind    o--o++--o+ ++o+++++--oo----- ----ooo++o++o--o--o-----
bury    +-----+o+ +o+o++-+-+-----o++ +o+---o-++o++o--o--oo++o++
mod      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
M/H      M      hM m mM M M M M M M M h m M M M M M M
prop     QVQLQQSGAE VAKPGASVKMSCKASGYTFT SYRMHWVKQAPGQGLEWIGYINPSTGYT
:Que     QVQLVQSGAE VKKPGSSVKVSKASGYTFT SYRMHWVRQAPGQGLEWIGYINPSTGYT

pos      60      70      80      90      100     110
EU      NYAQKFQGRVTITADESTNTAYMELSSLRSEDVAFYFCAG GYGIY SPEEYNGGLVTVSS
hH1     xyapxfqgRVTxttrdxSxntayMeLxSLrseDtAvYYCARxxxxxxxxxxwgqGtlvtVSS
TAC     EYNQKFQDKATLTADKSSSTAYMQLSSLTFEDSAVYYCAR GGGV FDYWGQGTTLTVSS
bind    -oooo+o+o+o+--o++o+o+++++-----o-----o+++++-----
bury    -o--o--++o--++o+o+o+---+---+---+---+---+---+---+---+---+---+---+---+
mod      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
M/H      M M M M M M M M M M h m h h h M M M M M M M M M M M
prop     EYNQKFQDKATLTADKSSSTAYMELSSLRSEDVAVYYCAR GGGV FDYWGQGTTLTVSS
Que      EYNQKFQDKATITADESTNTAYMELSSLRSEDVAVYYCAR GGGV FDYWGQGTTLTVSS

```

FIG. 12B

## INTERNATIONAL SEARCH REPORT

Internatio... Application No

PCT/US 94/05348

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/62 C07K15/12 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,92 22324 (XOMA CORPORATION) 23 December 1992 cited in the application see Examples 3 and 7 and claims. ---	1,9
A	PROC. NATL. ACAD. SCI. USA vol. 90, 1993 pages 457 - 461 M. BETTER ET AL.; 'Potent ant-CD5 ricin A immunoconjugates from bacterially produced Fab' and F(ab') <sub>2</sub> ' cited in the application see the whole document. ---	1,9
A	EP,A,0 506 124 (TANOX BIOSYSTEMS, INC.) 30 September 1992 see column 4, line 31 - column 6, line 11 and claims. --- -/--	1-8



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*Z\* document member of the same patent family.

Date of the actual completion of the international search

27 September 1994

Date of mailing of the international search report

21. 10. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Yeats, S

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/05348

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 05168 (RESEARCH DEVELOPMENT FOUNDATION) 18 March 1993 see claims. ---	1,9
P,X	WO,A,93 09130 (XOMA CORPORATION) 13 May 1993 cited in the application see Examples 10 and 12. ---	1,2, 13-16
P,X	J. BIOL. CHEM. vol. 269 , 1994 pages 9644 - 9650 M. BETTER ET AL.; 'Gelonin analogs with engineered cysteine residues form antibody immunoconjugates with unique properties' see abstract and Table IV. -----	1

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/05348

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9222324	23-12-92	AU-A- 2238292 CA-A- 2110799 EP-A- 0590067	12-01-93 23-12-92 06-04-94
EP-A-0506124	30-09-92	AU-B- 640863 AU-A- 1299292 JP-A- 5117164	02-09-93 15-10-92 14-05-93
WO-A-9305168	18-03-93	AU-A- 2547692 CA-A- 2115049 EP-A- 0602144 FI-A- 941048	05-04-93 18-03-93 22-06-94 04-03-94
WO-A-9309130	13-05-93	CA-A- 2122714	13-05-93

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**